Testicular Diffuse Large B-cell Lymphoma; Prognostic Clinical, Molecular, and Immunological Factors

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DOCTORAL DISSERTATION

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To Ville, Valtteri, Ilmari, and Lauri

Blue you sit so pretty west of the one
sparkles light with yellow icing, just a mirror for the sun.
Anthony Kiedis et al.

Table of contents

List of original publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV):

I. Mannisto S, Vähämurto P, Pollari M, Clausen MR, Jyrkkiö S, Kellokumpu-Lehtinen PL, Kovanen P, Karjalainen-Lindsberg ML, d'Amore F, Leppä S. Intravenous but not intrathecal central nervous system-directed chemotherapy improves survival in patients with testicular diffuse large B-cell lymphoma. *Eur J Cancer*. 2019;115:27-36.

II. Leivonen SK, Pollari M, Bruck O, Pellinen T, Autio M, Karjalainen-Lindsberg ML, Mannisto S, Kellokumpu-Lehtinen PL, Kallioniemi O, Mustjoki S, Leppä S. T-cell inflamed tumor microenvironment predicts favorable prognosis in primary testicular lymphoma. *Haematologica*. 2019;104(2):338-346.

III. Pollari M, Pellinen T, Karjalainen-Lindsberg ML, Kellokumpu-Lehtinen PL, Leivonen SK and Leppä S. Adverse prognostic impact of T-bet+ regulatory T-cells in primary testicular lymphoma. *Submitted for publicatio*n.

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In addition, some unpublished data is presented.

Abbreviations

aaIPI	Age-adjusted International Prognostic Index
Ab	Antibody
ABC	Activated B-cell-like
AIDS	Acquired immune deficiency syndrome
AKT	Protein kinase B
APC	Antigen-presenting cell
B2M	beta-2-microglobulin
BCL2	B-cell lymphoma 2 gene
Bcl-2	B-cell lymphoma 2 protein
BCL6	B-cell lymphoma 6 gene
Bcl-6	B-cell lymphoma 6 protein
BCR	B-cell receptor
BTK	Bruton's tyrosine kinase
BL	Burkitt lymphoma
BTG1	B-cell translocation 1
BTLA	B- and T-lymphocyte attenuator
CAF	Cancer-associated fibroblast
CARD11	Caspase recruitment domain family member 11
CCL	Chemokine (C-C motif) ligand
CGCI	Cancer Genome Characterization Initiative
CD40L	CD40 ligand
CD62L	L-selectin
CD200R	CD2000 receptor
CDKN	Cyclin-dependent kinase inhibitor
cHL	Classical Hodgkin lymphoma
CHOP	Cyclophosphamide-doxorubicin-vincristine-prednisone
COO	Cell-of-origin
CNS	Central nervous system
CR	Complete remission
CREBBP	CREB-binding protein
CT	Computer tomography
CTL	Cytotoxic T-lymphocyte
CTL1	Choline transporters-like protein 1
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	Chemokine (C-X-C motif) ligand
Cy	Cyanine
DC	Dendritic cell
DLBCL	Diffuse large B-cell lymphoma
DSS	Disease-specific survival
EBF1	EBF transcription factor 1
ECOG	Eastern Cooperative Oncology Group
EFS	Event-free survival
EGFR	Epidermal growth factor receptor
FasL	Fas ligand
FDG-PET-CT	Fluorodeoxyglucose positron emission tomography-computed tomography

FFPE	Formalin-fixed, paraffin-embedded
FL	Follicular lymphoma
FOXP1	Forkhead box P1
FOXP3	Forkhead box P3
GC	Germinal center
GCB	Germinal center B-cell-like
GEP	Gene expression profiling
GFP	Green fluorescent protein
GrA	Granzyme A
GrB	Granzyme B
GSK-3β	Glycogen synthase kinase 3 beta
HD-Ara-C	High dose cytarabine
HD-Mtx	High dose methotrexate
HIF1A	Hypoxia-inducible factor-1 alpha
HIV	Human immunodeficiency virus
HL	Hodgkin lymphoma
HLA	Human leukocyte antigen
HRS	Hodgkin-Reed-Sternberg
ICOS	Inducible T-cell co-stimulator
Ig	Immunoglobulin
IgVH	Immunoglobulin variable region heavy chain
IHC	Immunohistochemistry
IκB-ζ	Inhibitor of nuclear factor kappaB Zeta
IL	Interleukin
IL-1R	Interleukin-1 receptor
INFγ	Interferon gamma
IPI	International Prognostic Index
IRF4	Interferon regulatory factor 4
IT	Intrathecal
iT_{reg}	Induced regulatory T-cell
IV	Intravenous
KLHL14	Kelch-like protein 14 gene
KLRG1	Killer cell lectin-like receptor G1
LAG-3	Lymphocyte-activation gene 3
LDH	Lactate Dehydrogenase
Lgr4	Leucine-rich repeat-containing G-protein-coupled receptor
LPS	Lipopolysaccharides
mAb	Monoclonal antibody
MALT	Mucosa-associated lymphoid tissue
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation gene 1
MCL	Mantle cell lymphoma
MDSC	Myeloid-derived suppressor cell
mIHC	Multiplex immunohistochemistry
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
MUM1	Multiple myeloma oncogene 1
MYD88	Myeloid differentiation primary response gene 88
NCCN	National Comprehensive Cancer Network

NF1	Neurofibromatosis type 1 gene
NF-κB	Nuclear factor kappaB
NFKBIZ	NF-κB inhibitor Zeta
NHL	Non-Hodgkin lymphoma
NK	Natural killer
Non-GCB	Non-Germinal center B-cell-like
NOS	Not otherwise specified
nT_{reg}	"natural" regulatory T-cell
OS	Overall survival
PAX5	Paired box protein 5 gene
PCNSL	Primary central nervous system lymphoma
PD-1	Programmed cell death-1
PD-L1	Programmed cell death ligand 1
PD-L2	Programmed cell death ligand 2
PFS	Progression-free survival
PIK3	Phosphoinositide 3-kinase
PMBL	Primary mediastinal B-cell lymphoma
PRDM1	PR domain containing 1
Pros1	Tumor-secreted protein S
PT-DLBCL	Primary testicular diffuse large B-cell lymphoma
PTL	Primary testicular lymphoma
RFS	Relapse-free survival
R-CHOP	Rituximab-cyclophosphamide-doxorubicin-vincristine-prednisone
R-IPI	Revised International Prognostic Index
RNA	Ribonucleic acid
SGK1	Serum/glucocorticoid regulated kinase 1 gene
SHM	Somatic hypermutation
SIRPα	Signal regulatory protein alpha
Stat	Signal transducer and activator of transcription
TAM	Tumor-associated macrophage
T-bet	T-box expressed in T-cells
TCR	T-cell receptor
T-DLBCL	Testicular diffuse large B-cell lymphoma
TGF-β	Transforming growth factor beta
Th	T helper
Tfh	T follicular helper
Tf_{reg}	Follicular regulatory T-cell
TIL	Tumor-infiltrating lymphocyte
TIM-3	T-cell immunoglobulin and mucin domain containing-3
TLR	Toll-like receptor
TMA	Tissue microarray
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TNFα	Tumor necrosis factor alpha
TNFβ	Tumor necrosis factor beta
TNFR	Tumor necrosis factor receptor
T_{reg}	Regulatory T-cell

Abstract

Primary testicular lymphoma (PTL) is a rare lymphoma entity presenting in an immune-privileged site of the testis. The most common histology is activated B-cell-like (ABC) or non-germinal center B-cell-like (non-GCB) phenotype diffuse large B-cell lymphoma (DLBCL), often occurring with an aggressive clinical behavior and a relatively high relapse rate. Due to the rareness of the disease, no randomized clinical trials have been conducted in testicular DLBCL (T-DLBCL), and the currently recognized standard of care is based on retrospective analysis and few phase II trials.

During the last years, tumor microenvironment (TME) and tumor-related immunity have been the focus of many tumor biology studies, and the emergence of targeted therapies and checkpoint inhibitors has significantly modulated the field of cancer therapies. The role of distinct tumor-infiltrating immune cell subtypes and their immunophenotypes has not, however, been thoroughly studied in T-DLBCL.

The aims of this study were to evaluate the efficacy of different treatment modalities among T-DLBCL patients identified from the pathology databases of three university hospitals in Finland and the Danish lymphoma registry as well as to characterize tumor-infiltrating immune cell subtypes, their phenotypes and distribution in the TME, and to associate the findings with known clinical and biological risk factors and survival of patients with T-DLBCL.

The efficacy of different treatment modalities was evaluated by comparing the patient outcomes of altogether 189 Finnish and Danish T-DLBCL patients. Among these patients, intravenously (IV) administered central nervous system (CNS) -targeted chemotherapy and treatment of the contralateral testis translated into significantly longer patient survival, especially among elderly patients. Intrathecally (IT) administered CNS prophylaxis had no effect on the patient outcome, and rituximab seemed to be beneficial among high-risk (International Prognostic Index (IPI) score 3-5) T-DLBCL patients. In our patient material, the overall CNS relapse rate was low, and no differences in the risk of CNS relapse could be observed between patients treated with different treatment modalities. However, the use of IV CNS-targeted chemotherapy significantly improved the systemic control of lymphoma.

Tumor-infiltrating immune cell subtypes, their phenotypes and distribution in the TME were studied using gene expression analysis and multiplex immunohistochemistry (mIHC) on tissue microarray (TMA) slides of 60–79 Finnish T-DLBCL patients. We observed that higher expression of 121 T-lymphocyte signature genes enriched for T-lymphocyte markers associated with significantly longer survival of T-DLBCL patients. The association with survival was especially evident among rituximab-treated T-DLBCL patients and could also be seen in an independent cohort of 96 primary DLBCL patients.

Altogether, we observed a great variation in the distribution of distinct tumor-infiltrating lymphocyte (TIL) phenotypes. Higher proportions of $CD3^+$ TILs in general, $CD4^+$ TILs, and $CD8^+$ cytotoxic T-lymphocytes (CTLs) translated into better outcome. Higher proportions of programmed cell death-1 (PD-1) expressing $CD4^+$ TILs and CTLs associated with significantly longer patient survival. Despite the overall favorable prognostic impact of high TIL content, a subpopulation of $FoxP3^+T\text{-}bet^+$ double-positive regulatory T-cells (T_{reg}s) could be identified to have a significant adverse effect on the patient outcome.

We were able to show that a large proportion of both tumor-associated macrophages (TAMs) and lymphoma cells expressed programmed cell death ligand 1 (PD-L1), and higher proportions of $PD\text{-}L1^+CD68^+$ TAMs associated with longer survival. The content of $PD\text{-}L1^+CD68^+$ TAMs correlated with the content of $PD\text{-}1^+CD4^+$ TILs and $PD\text{-}1^+$ CTLs, and the proportion of $PD\text{-}L1^+CD68^-$ cells did not have an effect on the outcome of T-DLBCL patients.

In conclusion, our results support the use of the currently recognized standard of care with anthracycline-based immunochemotherapy and IV CNS-targeted chemotherapy with prophylactic treatment of the contralateral testis as the first-line treatment of patients with T-DLBCL. The results from our biological studies provide novel information on tumor-infiltrating immune cell subtypes and their phenotypes, their association with known clinical and biological risk factors as well as survival among patients with T-DLBCL. Future studies should focus on further characterizing the functional role of distinct tumor-infiltrating immune cells as well as on evaluating new, less toxic, targeted therapies in T-DLBCL also for elderly, more fragile patients.

Tiivistelmä suomeksi

Primaari kiveslymfooma on harvinainen ja kliiniseltä käyttäytymiseltään aggressiivinen lymfooma-alatyyppi, johon liittyy korkea taudin uusiutumisriski. Se esiintyy veri-kivesesteen suojaamassa kiveksessä ja edustaa histologialtaan yleisimmin aktivoituneen B-solun kaltaisen (ABC) tai ei-itukeskussoluperäisen (non-GCB) fenotyypin diffuusia suurisoluista B-solulymfoomaa. Taudin harvinaisuudesta johtuen primaarin kiveslymfooman hoidoista ei ole tehty satunnaistettuja kliinisiä hoitotutkimuksia, vaan yleisesti hyväksytty standardihoito perustuu takautuviin tutkimuksiin ja muutamaan faasin II tutkimukseen.

Viime vuosien aikana kasvainten mikroympäristö ja kasvaimiin liittyvä immuniteetti ovat olleet monien biologisten tutkimusten kohteena. Uusien kohdennettujen hoitojen ja immuunivasteen tarkistuspisteeseen vaikuttavien vasta-aineiden myötä syöpähoitojen kirjo onkin oleellisesti muuttunut. Kasvaimeen liittyvien immuunisolujen alaryhmien osuuksia ja kliinistä merkitystä primaarissa kiveslymfoomassa ei kuitenkaan ole aiemmin perusteellisesti tutkittu.

Tutkimuksen tavoitteina oli arvioida eri hoitomuotojen tehoa primaarin kiveslymfooman hoidossa ja selvittää kasvaimeen liittyvien immuunisolujen alaryhmien immuunifenotyyppejä ja jakautumista, sekä verrata tuloksia tunnettuihin kliinisiin ja biologisiin primaarin kiveslymfooman ennustetekijöihin sekä potilaiden hoitovasteeseen ja elossaoloon.

Eri hoitomuotojen tehoa arvioitiin vertailemalla 189 primaaria kiveksen diffuusia suurisoluista B-solulymfoomaa sairastaneen potilaan elossaolotietoja, jotka kerättiin kolmen suomalaisen yliopistosairaalan patologian tietokannoista sekä Tanskan lymfoomarekisteristä. Näiden potilaiden keskuudessa suonensisäinen keskushermostoon kohdennettu ja vastakkaisen kiveksen suojaava hoito paransivat merkittävästi potilaiden ennustetta, erityisesti iäkkäämpien potilaiden keskuudessa. Aivoselkäydinnesteeseen annettu keskushermostoon kohdennettu hoito ei vaikuttanut potilaiden ennusteeseen. Rituksimabi vaikutti hyödyttävän erityisesti korkean uusiutumisriskin (International Prognostic Index (IPI) pisteet 3–5) potilaita. Keskushermostouusiutumien määrä oli aineistossamme kaiken kaikkiaan pieni, eikä eri hoitomuodoilla hoidettujen potilaiden välillä havaittu eroja keskushermostouusiutumien määrän suhteen. Suonensisäinen keskushermostoon kohdennettu hoito paransi kuitenkin merkittävästi lymfoomapotilaiden kokonaiselinaikaa.

Kasvaimeen liittyvien immuunisolujen alaryhmien immuunifenotyyppejä ja jakautumista tutkittiin käyttäen geenieksprессioanalyysiä ja multiplex-immunohistokemiaa (mIHK) hyödyntäen 60–79 suomalaisen kiveksen diffuusia suurisoluista B-solulymfoomaa sairastaneen potilaan kasvainkudoksen mikrosirunäytteitä. Havaitsimme korkean 121 T-lymfosyyttimerkkiaineiden suhteen rikastuneen geenijoukon ilmentymistason olevan selkeästi yhteydessä potilaiden pidempään elossaoloaikaan. Yhteys ennusteeseen oli havaittavissa erityisesti immunokemoterapiahoidetuilla potilailla, ja nähtävissä myös itsenäisessä 96 primaaria diffuusia suurisoluista B-solulymfoomaa sairastaneen potilaan aineistossa.

Havaitsimme kaiken kaikkiaan suurta vaihtelua kasvaimeen liittyvien T-solujen alatyypeissä eri potilaiden välillä. Yleisesti korkeampi $CD3^+$-kasvaimeen liittyvien T-solujen määrä sekä korkeampi $CD4^+$-T-solujen ja $CD8^+$-sytotoksisten T-solujen määrä johti potilaiden parempaan selviytymiseen. Myös korkeampi programmed cell death-1 (PD-1) -molekyyliä ilmentävien kasvaimeen liittyvien $CD4^+$-T-solujen sekä $CD8^+$-sytotoksisten T-solujen osuus oli yhteydessä potilaiden pidempään elossaoloon. Huolimatta yleisestä kasvaimeen liittyvien T-solujen korkeampaan määrään yhteydessä olevasta paremmasta ennusteesta löysimme $FoxP3^+T\text{-}bet^+$ kaksoispositiivisten säätelevien T-solujen alaryhmän, joka oli yhteydessä potilaiden merkitsevästi huonompaan selviytymiseen.

Pystyimme osoittamaan, että suuri osuus sekä kasvaimeen liittyvistä makrofaageista että lymfoomasoluista ilmentää ohjelmoidun solukuoleman ligandia 1 (PD-L1), ja korkeampi $PD\text{-}L1^+CD68^+$-kasvaimeen liittyvien makrofaagien määrä on yhteydessä potilaiden pidempään elossaoloon. $PD\text{-}L1^+CD68^+$-kasvaimeen liittyvien makrofaagien määrä korreloi $PD\text{-}1^+CD4^+$-kasvaimeen liittyvien T-solujen ja $PD\text{-}1^+$-sytotoksisten T-lymfosyyttien määrään, eikä $PD\text{-}L1^+CD68^-$-solujen osuudella ollut vaikutusta potilaiden selviytymiseen.

Yhteenvetona voidaan todeta, että tuloksemme puoltavat nykyisen antrasykliinipitoisen immunokemoterapian ja suonensisäisen keskushermostoon kohdennetun sekä vastakkaisen kiveksen suojaavan hoidon käyttöä kiveksen diffuusia suurisoluista B-solulymfoomaa sairastavien potilaiden ensilinjan hoitona. Biologisten tutkimustemme tulokset tuovat uutta tietoa kasvaimeen liittyvistä immuunisoluista, niiden alaryhmistä sekä niiden yhteyksistä tunnettuihin kliinisiin ja biologisiin ennustetekijöihin ja kiveksen diffuusia suurisoluista B-solulymfoomaa sairastavien potilaiden elossaoloon. Tulevien tutkimusten tulisi keskittyä selvittämään eri kasvaimeen liittyvien immuunisolujen toimintaa sekä arvioimaan uusia, aiempaa paremmin siedettyjä, kohdennettuja hoitoja kiveksen diffuusissa suurisoluisessa B-solulymfoomassa, myös vanhempien ja hauraampien potilaiden kohdalla.

1 Introduction

Lymphoma is a malignancy of the lymphocytes or the natural killer (NK) cells that can arise in the lymphoid organs such as lymph nodes and the spleen, or extranodal sites such as the bone marrow, the central nervous system (CNS) or testis. Lymphomas were first discovered by Thomas Hodgkin who studied enlarged lymph nodes and spleens of children and young adults in the 19th century [1]. The malignant Hodgkin lymphoma (HL) cells were recognized about 60 years later, and were later named Hodgkin-Reed-Sternberg (HRS) cells after Thomas Hodgkin, Dorothy Reed, and Carl Sternberg [2]. Since then, over 70 distinct lymphoma entities have been recognized, with the incidence ranging between 48–60/100,000/year in Finland during the last few decades (https://syoparekisteri.fi).

Lymphomas were first classified according to their morphology after the Rappaport classification [3]. With the constantly increasing information on lymphoma biology, the classification of lymphoid malignancies were thereafter revised by Kiel and Real before the introduction of the WHO lymphoma classifications, which have also been renewed and revised several times [4-6] To put it simply, lymphomas used to be divided into HLs and non-Hodgkin lymphomas (NHLs). This division, however, tells perhaps more about the history than the biology of the diseases, since some HLs are nowadays known to share features with other B-cell lymphom as [6]. Lymphomas can, nevertheless, be divided into HLs, B-cell lymphomas, and T-/NK cell lymphomas, according to the nature of the malignant cells, although the current WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, Revised 4th Edition no longer recognizes the definition of NHL [6]. B-cell lymphomas are much more common than T-/NK cell lymphomas, as only 10–15% of all lymphomas previously classified as NHLs represent peripheral T-cell lymphomas [7]. B-cell lymphomas as well as T-cell lymphomas can be divided according to their clinical behavior into aggressive and indolent diseases. Aggressive lymphomas are considered potentially curable diseases, whereas indolent lymphomas can have a very peaceful clinical behavior, with even spontaneous regression, and can therefore often be considered for a watch-and-wait policy instead of immediate oncological treatments.

Diffuse large B-cell lymphoma (DLBCL) is the most common aggressive B-cell malignancy covering 30–58% of all lymphomas previously classified as NHLs, with a significantly rising incidence from 0.75/100,000/year to 12/100,000/year in Finland during the 21st century (https://cancerregistry.fi) [8]. DLBCL, not otherwise specified (NOS) constitutes 25–35% of adult NHLs in developing countries, is more common in elderly individuals, and slightly more common in males [6]. Depending on the clinical and biological risk factors, 50–90% of DLBCL patients can be cured with standard treatment options. Extranodal presentation is a known clinical risk factor in DLBCL. Primary testicular lymphoma (PTL) is a rare and aggressive extranodal lymphoma arising primarily in the immune-privileged site of the testis and most commonly representing DLBCL histologically [9]. It accounts for about 1–2% of all lymphomas previously classified as NHLs and for about 5% of all testicular malignancies, yet being the most common testicular neoplasm in elderly men [9-14].

Due to the rareness of testicular DLBCL (T-DLBCL), no randomized phase III trials have been conducted and the internationally recognized standard of care is based on data from phase II trials [15,16]. The median 5-year overall survival (OS) and progression-free survival

(PFS) rates with standard treatment have been reported to be around 85% and 75%, respectively [15]. T-DLBCL has a high tendency of relapsing in other extranodal sites, most commonly the contralateral testis and the CNS, and especially relapses in the CNS have been associated with poor prognosis [17]. Treatment approaches have therefore been targeted with the aim of gaining both local and systemic effectiveness as well as prevention of a possible CNS relapse.

The tumor microenvironment (TME) consists of different host immune cells, stromal cells, blood vessels, cell matrix, and numerous cytokines, chemokines, and exosomes [18]. The role of host immunity in tumor evolution and tumor growth inhibition was already recognized several decades ago. However, only the recent increasing understanding of the complexity of the host-related factors and immune escape has led to major advancements in the medical field, as checkpoint inhibitors and other immune modulators have been shown to be effective in several malignancies including lymphomas [18-21].

The studies in this thesis were planned and conducted with the aim of gaining novel information on the TME of T-DLBCL. The role of tumor-infiltrating lymphocytes (TILs) and tumor-associated macrophages (TAMs) and the expression of checkpoint molecules as well as T-cell exhaustion in T-DLBCL had not been thoroughly studied before and were the main focus of this thesis. Additionally, this thesis provides important information on the treatment outcomes of a large retrospectively analyzed Nordic T-DLBCL series, increasingly important in the lack of randomized phase III trials in this rare and unique lymphoma entity.

2 Review of the literature

2.1 B-cell differentiation and lymphoma pathogenesis

Naïve B-cells are produced by the bone marrow and differentiate into memory B-cells and antibody (Ab)-producing plasma cells essential for long-lived humoral immunity [22]. The migration, activation, differentiation, and proliferation of B-cells is regulated by several different mechanisms including antigens and pathogens, other immune cells, as well as different cytokines and chemokines. In this chapter, the main principles of B-cell evolution are reviewed, leaving out a more thorough description of all the mechanisms involved.

Naïve lymphocytes home from bone marrow to lymph nodes and spleen in high endothelial venules [23,24]. To be activated in the lymph nodes, B-cells need to encounter antigens either presented to B-cells by antigen-presenting cells (APCs), mainly macrophages and dendritic cells (DCs), or in soluble form; both in the B-cell follicles of the lymph node cortex [25]. The antigens are bound by B-cell receptors (BCRs), internalized, and again presented by the B-cells via major histocompatibility complex (MHC) class II to further induce T-cell activation as well as B-cell differentiation and proliferation [26]. The differentiation of activated B-cells into memory B-cells can be either germinal center (GC)-derived or GC-independent whereas differentiation into plasma cells mainly occurs in GCs [22,27,28].

GC-derived differentiation is conducted in the GC of the B-cell follicle where B-cells first proliferate in the 'dark zone', then differentiate in the 'light zone', and again re-enter the 'dark zone' for further rounds of proliferation and somatic hypermutation (SHM), BCR-affinity-based selection, or apoptosis [29-31]. Antibodies produced by mature B-cells are coded by immunoglobulin heavy chain variable region (IgVH) genes, and SHM of the IgVH genes in combination with affinity-based selection is essential for the development of high-affinity Ab-producing B-cells [29,31-34]. The GC-independent differentiation occurs outside the GC in a pre-GC phase regulated by T follicular helper (Tfh) cells, also involved in initiating the differentiation of some B-cells into plasma cells [27,28,33]. Additionally, interactions between B-cells and T-cells are essential for proper B-cell function and Ab production. T-cell help is required for gaining entry of B-cells into the GC pathway of memory B-cell differentiation as well as for later memory B-cell proliferation and differentiation into plasma cells following antigen recall [26,27,35].

Many B-cell lymphomas show similarities to normal B-cell developmental stages according to their immunophenotype, histological appearance, and gene expression profile. In lymphomas, however, the normal signaling pathways can be altered, leading to constitutively activated pro-survival pathways [36]. Increasing knowledge on genetic alterations as well as modifications in the intracellular pathways and the TME has consistently led to better understanding of lymphoma pathogenesis [36-39].

Some B-cell lymphoma entities can be associated with a specific B-cell developmental stage: the majority of Burkitt lymphomas (BLs), follicular lymphomas (FLs), and germinal center B-cell-like (GCB) DLBCLs have gene expression profiles most similar to B-cells of the GC 'light zone', although some BLs seem to be more close to cells from the 'dark zone' [39-41]; activated B-cell-like (ABC) DLBCL cells have experienced the GC phase and are closest to plas-

mablast stage of B-cell development [36,39,40]; mantle cell lymphoma (MCL) cells resemble naïve B-cells but a minority of them show SHM, indicating that they have undergone the GC phase [42]; and mucosa-associated lymphoid tissue (MALT) lymphoma cells resemble post-GC B-cells of the outer layer of B-cell follicle named the marginal zone [43]. A summary of B-cell development and the origin of malignant cells in distinct lymphoma entities is shown in Figure 1. The cell-of-origin (COO) in DLBCL and T-DLBCL is reviewed more closely in section 2.3.1.

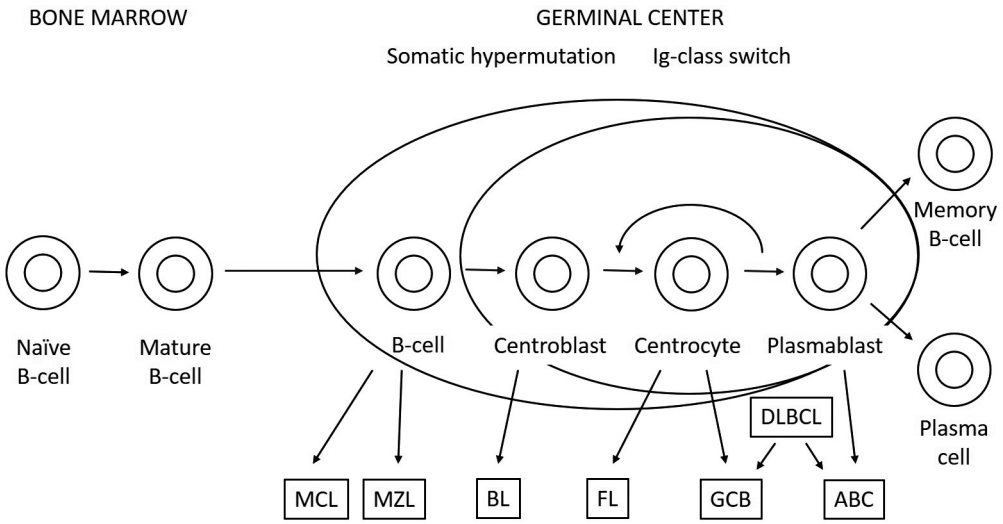

Figure 1. **B-cell development and the origin of malignant cells in distinct lymphoma entities.** In the normal GC-derived differentiation, B-cells proliferate in the 'dark zone' of the GC and differentiate in the 'light zone', and again re-enter the 'dark zone' for further rounds of proliferation and SHM, BCR-affinity-based selection, or apoptosis. Some B-cell lymphoma entities can be associated with a specific B-cell developmental stage according to their immunophenotype, histological appearance, and gene expression profile. The majority of BLs, FLs, and GCB phenotype DLBCLs have gene expression profiles most similar to B-cells of the GC 'light zone'; ABC phenotype DLBCL cells have experienced the GC phase and are closest to plasmablast stage of B-cell development, whereas MCL cells resemble naïve B-cells and MALT lymphoma cells resemble post-GC B-cells of the outer layer of the B-cell follicle named the marginal zone. Adjusted from Pasanen et al. 2017 [44].

The most important signaling pathways in lymphoma pathogenesis include nuclear factor kappaB (NF-κB), BCR, and Toll-like receptor (TLR) signaling pathways [36]. Activation of NF-κB pathway promotes cell survival by several distinct mechanisms as well as antagonizes apoptotic action of chemotherapy and is considered a hallmark of ABC-DLBCL (discussed more closely in section 2.3.1) [36,45,46]. The activation of NF-κB signaling pathway can be induced by a multidomain signaling adapter caspase recruitment domain family member 11 (*CARD11*) or alternatively, by Bruton's tyrosine kinase (BTK), a tyrosine kinase functioning downstream of BCR [36,47-49]. Furthermore, *CARD11* functions through regulating downstream factors such as mucosa-associated lymphoid tissue lymphoma translocation gene 1 (*MALT1*) protein product MALT1, another significant growth-promoting protein that potentiates NF-κB signaling by activating further downstream factors and by inactivating negative regulators of the signaling pathway [50-54].

As previously described, BCR is essential in B-cell development, antigen-driven clonal selection, and humoral immunity. It consists of signal transducing immunoglobulin (Ig) α (CD79A) and Igβ (CD79B) subunits that regulate BCR surface expression, internalization, and trafficking, while antigen specificity of BCR is provided by the surface Ig [55,56]. After BCR clustering and antigen encounter, the subunits transmit signals to several downstream signaling pathways including the NF-κB pathway [56,57].

TLR activation has a role in normal peripheral B-cell responses and autoimmune humoral responses [55,58-60]. TLRs regulate various downstream signaling pathways, including the NF-κB pathway [61,62]. In lymphoma pathogenesis, upregulation of TLR signaling pathway is mainly mediated by adapter protein MYD88, *MYD88* mutations being oncogenic gain-of-function mutations [63]. *MYD88* is known to be essential in ABC-DLBCL as knockdown of *MYD88* has been shown to be toxic in ABC-DLBCL cell lines [63]. Additionally, BCR signaling and *MYD88*/TLR-mediated signaling have been demonstrated to collaborate in a synergic manner in autoimmune diseases and have also been suggested to have synergistic function in lymphoma pathogenesis [36,60,63].

Further modifications in distinct intracellular signaling pathway mechanisms are beyond the scope of this thesis and are therefore not discussed more thoroughly in this literature review. The role of TME is reviewed in section 2.4.

2.2 Lymphoma classification

Lymphoma entities are classified according to the World Health Organization (WHO) classification of Tumours of Haematopoietic and Lymphoid Tissues. The latest version of the WHO classification is the Revised Fourth Edition published in 2017 [6]. The classification defines the diagnostic criteria and genetic/molecular landscape of lymphoma entities as well as reviews the latest most important advancements in the field of lymphoma biology research, discussing also the therapeutic approaches in lymphoid neoplasms. The classification is a consensus among hematopathologists, geneticists, and clinicians, and describes over 90 different entities of which aggressive mature B-cell lymphomas are listed in Table 1, according to the latest revised Fourth Edition [6].

DLBCL is the most common lymphoma entity and the most common histology of PTL. All PTL patients in this thesis represent T-DLBCL and the subsequent literature review is therefore focused on DLBCL in general and T-DLBCL.

Table 1. Aggressive mature B-cell neoplasms according to WHO classification of Tumours of Haematopoietic and Lymphoid Tissues, the revised Fourth Edition.

Diffuse large B-cell lymphoma (DLBCL), NOS
Germinal center B-cell subtype
Activated B-cell subtype
T-cell/histiocyte-rich large B-cell lymphoma
Primary DLBCL of the CNS
Primary cutaneous DLBCL, leg type
EBV+ DLBCL, NOS
EBV+ mucocutaneous ulcer
DLBCL associated with chronic inflammation
Fibrin-associated diffuse large B-cell lymphoma
Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
ALK+ large B-cell lymphoma
Plasmablastic lymphoma
Primary effusion lymphoma
HHV8+ DLBCL, NOS
Burkitt lymphoma
Burkitt-like lymphoma with 11q aberration
High-grade B-cell lymphoma
High-grade B-cell lymphoma, with *MYC* and *BCL2* and/or *BCL6* rearrangements
High-grade B-cell lymphoma, NOS
B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classic Hodgkin lymphoma

Provisional entities listed in italics. EBV, Epstein-Barr virus; ALK, Anaplastic Lymphoma Kinase; HHV8, Human herpesvirus 8. Adjusted from Swerdlow et al. 2017 [6].

2.2.1 Diffuse large B-cell lymphoma (DLBCL)

By definition, DLBCL is a neoplasm of large B lymphoid cells with nuclear size equal to or exceeding normal macrophage nuclei or more than twice the size of a normal lymphocyte, that has a diffuse growth pattern [6]. The neoplastic cells typically express pan-B-cell markers such as CD19, CD20, CD22, CD79a, and PAX5, but may also lack one or more of these [6]. Surface and cytoplasmic Ig, most commonly IgM, can be demonstrated in 50–75% of the cases and the neoplastic cells express CD5 in 5–10% of the cases [6]. Proliferation index Ki-67 is high, usually varying between 40% and >90% [6]. Expression of p53 occurs in 20–60% of the cases, being more common than mutations of *TP53*, further suggesting a possible upregulation of wildtype *TP53* in some cases [6]. Expression of c-Myc, Bcl-2, and Bcl-6 varies and is discussed in more detail later in sections 2.3.1 and 2.3.2.

The WHO classification of Tumours of Haematopoietic and Lymphoid Tissues, the revised Fourth Edition, subdivides DLBCL into morphological variants, molecular subtypes and distinct disease entities according to morphological, biological, and clinical studies [6]. Additional entities with features intermediate between DLBCL and BL (High-grade B-cell lymphoma, with *MYC* and B-cell lymphoma 2 *(BCL2)* and/or B-cell lymphoma 6 (*BCL6*) rearrangements; and high-grade B-cell lymphoma, NOS) or DLBCL and classical HL (cHL) (B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma) are recognized, Table 1 [6].

DLBCL, NOS comprises all DLBCL cases that do not follow any of the listed specific DLBCL diagnostic categories, Table 1 [6]. It can be further divided according to the COO classification dividing DLBCLs into GCB and ABC/non-GCB phenotypes [6]. The COO separates two entities with different chromosomal alterations, activation of signaling pathways, and clinical outcome, and is addressed in more detail in section 2.3.1. [6,39,64,65].

High-grade B-cell lymphoma, with *MYC* and *BCL2* and/or *BCL6* rearrangements, the so-called 'double-hit' or 'triple-hit' lymphoma, is a highly aggressive lymphoma entity discussed in more detail later in section 2.3.2 focusing on Bcl-2, Bcl-6 and c-Myc. Other DLBCL entities mentioned in the WHO classification are significantly more uncommon and only very rarely presented in the testis, and are therefore not further discussed in this review.

2.2.2 Primary testicular diffuse large B-cell lymphoma (T-DLBCL)

The majority of PTL cases (75–90%) represent DLBCL, most commonly ABC/non-GCB phenotype, as described in more detail in section 2.3.1. [9,66-71]. The WHO classification of Tumours of Haematopoietic and Lymphoid Tissues, the revised Fourth Edition, does not yet recognize T-DLBCL as its own distinct entity, though increasing evidence shows marked differences in the biology of DLBCL of the testis, many of them similar to primary DLBCL of the CNS (primary central nervous system lymphoma, PCNSL) [72-74], another DLBCL entity arising in an immune-privileged site and recognized as its own entity in the WHO classification [6].

Other lymphoma entities with reported testicular involvement are FL, MCL, immunoblastic NHL, diffuse mixed cell NHL, T-cell lymphoma, BL, T-/NK cell lymphoma, and plasmacytoma [70,75-80]. Since all the patients studied in this thesis represented T-DLBCL, these other rare PTL subtypes are not further discussed in this literature review.

2.2.2.1 Epidemiology and etiology

T-DLBCL is a rare lymphoma entity with reported annual incidence of 0.09–0.26 per 100,000 population [9-14]. It accounts for about 1–2% of all lymphomas previously classified as NHLs and about 5% of all testicular malignancies [9-14]. However, it is the most common testicular neoplasm in elderly men, with the previously reported median age at diagnosis varying from 59 to 68 years [17,66,67,81-83]. In non-immune competent individuals, such as patients with HIV/AIDS, T-DLBCL may also arise in younger patients [84]. Additionally, T-DLBCL has been reported as the most common bilateral testicular malignancy with either concurrent or subsequent bilateral testicular involvement [10,12,83,85,86].

Little is known about the etiology of T-DLBCL, although HIV infection is a recognized risk factor for aggressive lymphomas and primary extranodal lymphomas [84,87]. Testis is, however, an immune-privileged site with several different mechanisms designed to protect the developing gametocytes [9,88-90]. This may serve T-DLBCL a distinct milieu ideal for developing an immune escape phenotype similar to PCNSL, another entity arising in an immune-privileged site, as T-DLBCL and PCNSL are known to share common genetic features such as high levels of IgVH gene SHM and common loss of human leukocyte antigen (HLA) gene expression, discussed in more detail in section 2.3.3 [74,91-94].

2.2.2.2 Symptoms, diagnosis, and staging

Most commonly T-DLBCL presents with a firm, painless mass in a testis. The median tumor size at presentation is reported to be 4–6 cm with no preference for either side [13,69,95]. T-DLBCL is the most common bilateral testicular malignancy with either concurrent or subsequent involvement of the contralateral testis; synchronous bilateral involvement being described in 6–15% of the cases [11,13,67,69,83,86]. B-symptoms (fever, loss of weight, sweating) are rare and indicate a disseminated disease, present in 20–30% of the patients at diagnosis [17,67,69,83,96].

T-DLBCL has a high relapse rate and the recurrence often occurs in the contralateral testis or, most commonly, in the CNS [17,66,69,81,85]. Especially the involvement/recurrence of the CNS has been associated with a worse prognosis, and relapses in the CNS have been reported in up to 30% of the cases within 1–2 years from diagnosis [17,97]. Other reported extranodal sites of relapse are skin, subcutaneous tissue, lung, and Waldeyer ring, and late relapses even up to 10 years after primary diagnosis have been described [17,67,81,85,98].

The histopathological diagnosis of T-DLBCL is obtained from a tissue sample of the involved testis, according to the latest WHO classification of Tumours of Haematopoietic and Lymphoid Tissues [6]. Ultrasound of the contralateral testis and a contrast-enhanced computer tomography (CT) of the whole body and neck as well as bone marrow biopsy are required for staging [97]. Additional 18-fluorodeoxyglucose positron emission tomography-computed tomography (18-FDG-PET-CT) is highly recommended as it is more sensitive in detecting possible other extranodal lymphoma lesions [99]. Magnetic resonance imaging (MRI) of the brain and cytological and flow cytometric analysis of the cerebrospinal fluid are also recommended

as part of lymphoma staging, although they are often carried out only if indicated by clinical symptoms or findings, as are further targeted examinations such as consultation of an ophthalmologist [97].

The stage of T-DLBCL is commonly classified by the Ann Arbor lymphoma staging, Table 2 [96,97].

Table 2. Ann Arbor lymphoma stage classification.

Stage	Description
I	Involvement of a single lymphatic region (I) or localized involvement of single extranodal organ or site (IE)
II	Involvement of two or more lymphatic regions on the same side of the diaphragm (II) or localized involvement of a single extranodal organ or site and one or more lymphatic regions on the same side of the diaphragm (IIE)
III	Involvement of lymphatic regions on both sides of the diaphragm
IV	Diffuse or disseminated involvement of one or more extranodal organs with or without lymphatic involvement
Adjusted from Carbone et al. 1971 [96].	

2.2.2.3 Clinical prognostic factors

Some clinical features such as higher age at diagnosis and advanced stage disease (stage III-IV) are known to associate with worse prognosis in patients with DLBCL [100]. The same clinical prognostic factors have been shown to predict the outcome of T-DLBCL patients [66-69,71,82,83,85,101-103]. Based on these findings, the prognosis of patients with DLBCL and T-DLBCL can be estimated according to the International Prognostic Index (IPI) or the modified age-adjusted IPI (aaIPI), Tables 3 and 4, respectively [8,100,104].

Table 3. International Prognostic Index.

Risk group	IPI score	3-year OS, (95% CI), %
Low	0 or 1	91 (89–94)
Intermediate low	2	81 (76–86)
Intermediate high	3	65 (58–73)
High	4 or 5	59 (49–69)
1 score for each: Stage III-IV, elevated Lactate Dehydrogenase, age over 60 years at diagnosis, ECOG performance status 3-4, more than 1 extranodal lymphoma site. Adjusted from Ziepert et al. 2010 [104].		

Table 4. Age-adjusted International Prognostic Index.

Risk group	aaIPI score	3-year OS, (95% CI), %
Low	0	98 (96–100)
Intermediate low	1	92 (87–95)
Intermediate high	2	
High	3	75 (66–82)
1 score for each: Stage III-IV, elevated Lactate Dehydrogenase, ECOG performance status 3-4. Adjusted from Tilly et al. 2015 [8].		

Since the original IPI was determined in the pre-rituximab era, modifications of IPI have been launched for patients treated with immunochemotherapy. The revised IPI (R-IPI) uses the same clinical prognostic factors as the original IPI to identify three distinct prognostic subgroups within immunochemotherapy-treated DLBCL patients and has been reported to predict the outcome of these patients better than the original IPI, Table 5 [105]. The National Comprehensive Cancer Network IPI (NCCN-IPI) similarly uses the clinical prognostic factors of the original IPI to divide immunochemotherapy-treated DLBCL patients into four different subgroups with discriminated prognosis, Table 6 [106]. Since the use of these modified prognostic indexes has not been validated in larger trials, and the original IPI has been validated as prognostic also in the rituximab era, the decision-makingin in daily clinical practice is, nonetheless, still based on the original IPI [104].

Table 5. Revised International Prognostic Index.

R-IPI	Number of IPI factors	4-year PFS, %	4-year OS, %
Very good	0	94	94
Good	1, 2	80	79
Poor	3, 4, 5	53	55
IPI, International Prognostic Index; PFS, Progression-free survival; OS, Overall survival. Adjusted from Sehn et al. 2007 [105].			

Table 6. National Comprehensive Cancer Network International Prognostic Index.

Factor	Score	NCCN-IPI	Total Score	5-year PFS, %	5-year OS, %
Age, years		Low	0, 1	91	96
> 40 to ≤60	1	Low-intermediate	2, 3	74	82
>60 to ≤75	2	High-intermediate	4, 5	51	64
over 75	3	High	≥6	30	33
LDH-ratio					
>1 to ≤3	1				
over 3	2				
Ann Arbor stage III-IV	1				
Extranodal disease	1				
ECOG performance status ≥2	1				

PFS, Progression-free survival; OS, Overall survival; LDH, Lactate Dehydrogenase. Adjusted from Zhou et al. 2014 [106].

2.2.2.4 Treatments

Besides reaching a complete remission, the intention of T-DLBCL treatment has been to prevent relapses of the contralateral testis and the CNS [17]. Due to rareness of the disease, there are no randomized phase III trials and the internationally recognized standard of care mainly relies on two prospective phase II trials [15,16]. Based on these trials, the backbone of T-DLBCL treatment is considered to be orchiectomy followed by immunochemotherapy with six cycles of R-CHOP (rituximab-doxorubicin-cyclophosphamide-vincristine-prednisone) or R-CHOP-like regimen given every 21 days [97]. The addition of CNS prophylaxis with intravenously (IV) administered CNS-penetrating chemotherapy such as high dose methotrexate (HD-Mtx) or high dose cytarabine (HD-Ara-C) and/or intrathecal (IT) chemotherapy as well as irradiation or excision of the contralateral testis are recommended [15,16,85,97]. Supporting the data from phase II trials, the use of anthracycline-based chemotherapy has been shown to improve the outcome of T-DLBCL patients also in retrospective series [85]. Correspondingly and in line with findings on DLBCL in general, anti-CD20 monoclonal antibody (mAb) rituximab has been reported beneficial in T-DLBCL in retrospective analysis [16,102]. The use of CNS-targeted chemotherapy is recommended although data on whether it should be administered IT or IV is scarce, and the risk of CNS relapse has not decreased in the rituximab era [102]. On the contrary, data on irradiation of the contralateral testis reducing the risk of recurrence of the contralateral testis is more convincing [15,16,101].

2.3 Biology of DLBCL and T-DLBCL

2.3.1 Cell of origin (COO)

The original COO classification, a study based on gene expression profiling (GEP) of 3,186 genes with known importance to lymphocyte and/or cancer biology, was published in 2000 separating two distinct DLBCL entities with different clinical outcomes and different gene expression patterns, indicating separate stages of B-cell differentiation [39]. Tumors with GCB phenotype were shown to express genes characteristic of GC B-cells whereas tumors with ABC phenotype expressed genes that are normally induced during *in vitro* activation of peripheral blood cells [39]. Patients with GCB phenotype were reported to have significantly longer OS compared to patients with ABC phenotype, and the findings have later been confirmed by other studies, although trials not finding an association with COO and survival have also been reported [39,40,107-109].

Since the original COO publication, an additional third molecular subgroup of primary mediastinal large B-cell lymphoma has been recognized, while a small proportion of all DLBCLs still remains unclassified [6,110-112]. Furthermore, it has been recognized that the two original COO subgroups differ in their activation of signaling pathways such as the NF-κB pathway, commonly activated in ABC phenotype [36,45,47,64]. The mutation status of distinct oncogenes also varies, with e.g. *CARD11*, myeloid differentiation primary response gene 88 (*MYD88*), and *MALT1* all being more commonly mutated in ABC phenotype [49,63,113]. Some of the main differences between ABC and GCB phenotypes are summarized in Table 7.

Table 7. Differences in disease characteristics between ABC and GCB DLBCL.

Characteristics	**ABC DLBCL**	**GCB DLBCL**
Median age at diagnosis	66 years	61 years
Immunophenotype	$CD20^+$, IRF^+, $FoxP1^+$, $CD10^-$, $BCL6^-$, $GCET1^-$, $LMO2^-$	$CD20^+$, $CD10^+$, $BCL6^+$, $GCET^+$, $LMO2^+$, $IRF4^-$, $FoxP1^-$
	Frequency in ABC-DLBCL	**Frequency in GCB-DLBCL**
Rearrangements		
BCL2	< 5%	40%
BCL6	25–30%	15%
MYC, single hit	< 5–8%	< 5–8%
CD274/PDCD1LG2 (coding PD-L1 and PD-L2)	Rare	Rare
Copy-number alterations		
9p24.1 gains/amplifications *(CD274/PDCD1LG2)*	Uncommon	Uncommon
18q21.3 gain/amplification (*BCL2*)	55%	15%
Recurrent mutations		
TP53	25%	20%
CARD11	10–15%	10–15%
CD79B	20-25%	Uncommon
MYD88	35%	Uncommon
Pathway perturbations		
NF-κB activation	Yes	No
PI3K/AKT	No	Yes
JAK/STAT signaling	Rare	Yes
Clinical features		
5-year PFS (R-CHOP)	40-50%	70-80%
Adjusted from Shaffer et al. 2012 and Swerdlow et al. 2017 [6,36].		

As GEP is not yet available as routine clinical practice in most laboratories, more feasible techniques such as immunohistochemistry (IHC) based algorithms have been studied in determining the COO [65,114-116]. The most commonly used IHC algorithm is the Hans algorithm which classifies COO into GCB or non-GCB phenotypes based on the expression of

CD10, Bcl-6, and MUM1, with significantly worse OS reported among patients with non-GCB phenotype [65]. The classification scheme used in the Hans algorithm is described in Figure 2. All IHC-based COO classifications may, however, have reproducibility problems between laboratories, and their reported prognostic value has been inconsistent [117,118]. They have also been reported not to recognize the 10–15% of tumors determined as unclassified by GEP [6]. Compared to IHC-based algorithms, newer methods such as assays with RNA transcript quantification of 14–20 genes have shown better reproducibility as well as separation of the two distinct COO prognostic subgroups corresponding to the original COO classification [119-123]. As they have not been validated in larger trials, the IHC-based classification of COO is still currently most commonly used in daily clinical practice, and the newer methods may serve as a promising alternative for COO classification in the future.

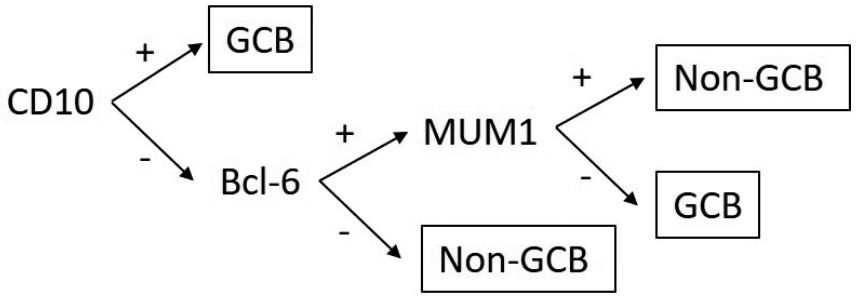

Figure 2. The Hans algorithm. The IHC-based Hans algorithm classifies COO into GCB or non-GCB phenotypes based on the expression of CD10, Bcl-6, and MUM1.

Adjusted from Hans et al. 2004 [65].

The majority of T-DLBCLs represent ABC/non-GCB DLBCL, defined with either GEP- or IHC-based algorithms, respectively, with the frequency varying between 60–96% according to proportion of patients with advanced stage disease and the IHC algorithm used [9,69,70,73,103,124-127]. The Hans algorithm has been the most commonly used COO classification in T-DLBCL studies [65]. It has been discussed whether IHC-based algorithms designed to classify nodal DLBCL can appropriately be applied to extranodal entities and whether GEP-based methods could more accurately classify COO in extranodal diseases [9,73,126]. According to the only GEP-based study in T-DLBCL, the proportion of ABC phenotype was reported to be 96% [126].

2.3.2 Bcl-2, Bcl-6, and c-Myc

BCL2 is an oncogene coding Bcl-2, a member of the Bcl-2 protein family, first discovered in FL in association with chromosomal translocation t(14;18) [128]. The Bcl-2 protein family can be divided into three separate groups according to the protein's structure and function: proteins that participate in inhibiting apoptosis, and two different groups with proteins that function as pro-apoptotic proteins but differ by structure [129]. Bcl-2 belongs to the anti-apoptotic group that regulates survival of cells by inhibiting the initiation of apoptosis [130-132]. It is known to be crucial for the pathogenesis of lymphoma and has been associated with multidrug-resistance in lymphoid malignancies [133-135]. High levels of Bcl-2 protein expression (more common in ABC-DLBCL) have been associated with poor prognosis in aggressive lymphomas, including DLBCL, while most of the studies showed no association to survival with *BCL2* rearrangements (more common in GCB phenotype DLBCL) [136-146]. The main functions of *BCL2* are summarized in Figure 3.

Overexpression of Bcl-2 was originally thought to result from translocation t(14;18) but later studies have described additional alternative mechanisms for the upregulation of the gene, and elevated gene expression and Bcl-2 protein levels have also been reported without (14;18) [128,147-150]. In T-DLBCL, nine patients with Bcl-2 positive B-cell NHL of the testis with no translocation t(14;18) were described in the 1990s [150]. Another study reported two Bcl-2 positive cases of primary cutaneous DLBCL, leg type, with testicular involvement at diagnosis, having identical clinicopathological and immunophenotypical features in their skin lesions as in the tumors of the testis, further interpreting both cases as T-DLBCL with cutaneous involvement [151]. In a larger T-DLBCL phenotype profiling study, 4% of the studied cases showed *BCL2* rearrangements and 15% of the cases an amplification of *BCL2*, while Bcl-2 was reported to be expressed in 70% of the studied T-DLBCL cases [127].

BCL6 is a transcription factor suppressing c-Myc and Bcl-2 expression as well as NF-κB pathway activation [152-155]. It is essential in normal GC reaction and B-cell development by preventing premature activation and differentiation of GC B-cells as well as by providing a suitable environment for further B-cell development [28,156,157]. It can block the terminal differentiation by repressing PR domain containing 1 (*PRDM1*) which encodes Blimp-1, the master regulator of plasmacytic differentiation, but can also enable cell-cycle progression by repressing cyclin-dependent kinase inhibitors *CDKN1A* and *CDKN1B* that induce cell cycle arrest [158-160]. Additionally, Bcl-6 is involved in the development of $CD4^+$ Tfh cells, essential in GC reaction [161,162]. The main functions of *BCL6* are summarized in Figure 3.

Deregulation of *BCL6* has been shown to be leukemogenic and to contribute to the pathogenesis of DLBCL [153,163]. Aberrations in *BCL6* have also been detected in T-DLBCL, but their functional role in T-DLBCL remains to be further studied [74]. Translocations of *BCL6* are shown to be more common in ABC/non-GCB DLBCL with contradictory results according to the association with survival when occurring either alone or in combination with *MYC* translocations [145,164-168]. Co-expression of Bcl-6 and c-Myc has, however, been described to translate into worse prognosis, but the association seems to be dependent of concurrent Bcl-2 and c-Myc co-expressions [164]. In T-DLBCL, Bcl-6 expression has been described to be low (5%) whereas *BCL6* has been reported to be frequently (29–48%) rearranged [74,127].

MYC is an oncogene promoting cell division by several distinct mechanisms including direct regulation of its target genes involved in cell proliferation and growth as well as control of messenger RNA, microRNAs, and apoptosis mediators [169-171]. Expression of *MYC* protein product c-Myc has been detected in up to 70% of all human malignancies [169]. It has been shown to be essential for the formation, maintenance, and proper function of GCs, and highly leukemogenic at several stages of B-cell maturation [157,169,172,173]. The main functions of *MYC* are summarized in Figure 3.

MYC rearrangements were discovered in BL in the 1980s [174]. They are known to be crucial for lymphoma pathogenesis in BL and in subsets of DLBCL and have been associated with poor prognosis in DLBCL [164,168,169,175-177]. *MYC* rearrangements occur in about 9% of DLBCL cases, and 60–80% of patients with a MYC translocation have been reported with concurrent Bcl-2 expression [168,175]. Furthermore, translocation of *MYC* seems to predict worse outcome of DLBCL patients especially when occurring concurrently with Bcl-2 expression [142]. Overexpression of c-Myc has been reported as an adverse prognostic factor among immunochemotherapy-treated DLBCL patients [144,146]. No association with a distinct COO subgroup has been shown with c-Myc protein expression whereas *MYC* rearrangements have been reported to be more common in GCB phenotype in some studies while other studies found no association with COO [142,164,168,175,178]. In T-DLBCL, only 6% of the cases have been reported with *MYC* rearrangements and 13% of the cases with positive c-Myc expression, and the role of *MYC* has not been thoroughly studied in T-DLBCL [127].

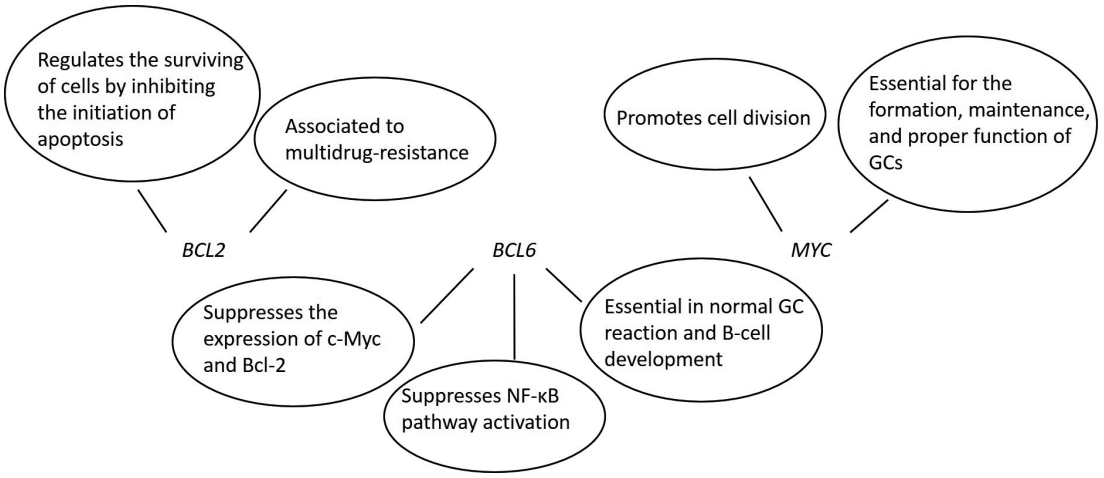

Figure 3. The functions of *BCL2, BCL6,* and *MYC*. The main functions of oncogenes *BCL2* and *MYC* as well as transcription factor *BCL6* are summarized in the figure.

When occurring together with translocation of *MYC*, translocations of *BCL2* or *BCL6* lead to a manifestation of aggressive double-hit lymphomas with reportedly significantly worse outcome, especially in DLBCL with concurrent translocations of *MYC* and *BCL2* [130,142,143,164,168,179-186]. About 60% of double-hit lymphomas have *MYC* and *BCL2* translocations while translocations of *MYC* and *BCL6* cover about 20% of double-hit lymphomas; about 20% of the cases have concurrent translocations of all *MYC*, *BCL2*, and *BCL6* (triple-hit lymphomas) translating into a most dismal outcome [176,181,182,187].

The majority of double-hit lymphomas represent GCB phenotype and over 65% of the cases have been reported with extranodal lymphoma involvement, most often involvement of the bone marrow or the CNS [144,164,180,181,185,186,188]. As majority of T-DLBCLs represent ABC phenotype, the frequency of *BCL2* and *MYC* rearrangements in T-DLBCL is comparable to nodal ABC-DLBCL (about 10% and 15%, respectively), while *BCL6* rearrangements seem less common in T-DLBCL (about 40% in T-DLBCL vs. 60% in nodal ABC-DLBCL) [73].

Cases with c-Myc and Bcl-2 or Bcl-6 protein expression have been named 'double-expressers', and cases with expression of M-myc, Bcl-2, and Bcl-6 correspondingly 'triple-expressers' [6]. In nodal DLBCL, double-expressers are more prevalent than double-hit lymphomas (20–30% vs. 5%, respectively) [109,142,144,176,178,189]. Double-expressers most commonly Represent ABC/non-GCB phenotype and associate with significantly increased risk of CNS relapse and shorter survival, contributing to the overall inferior prognosis of ABC phenotype [109,142,145,178, 189-192]. The frequency of double-expressers among T-DLBCL cases has, however, been reported to be markedly lower, about 13% [127].

2.3.3 Genetic landscape

Besides the COO-based division into ABC or GCB phenotype, recent genetic studies on DLBCL have been able to identify other additional phenotypically distinct DLBCL subtypes with different genetic aberrations, gene-expression signatures, and responses to immunochemotherapy [146,193,194]. Regarding aberrations in individual genes, mutations in driver genes such as *MYC* and *CD79B* have been associated with poor survival whereas mutations in *NF1* and *SGK1* have been shown to associate with favorable outcome in DLBCL [146].

The genetic features generally associated with ABC-DLBCL are summarized in Table 7. They include increased activation of the NF-κB pathway through mutations of *CARD11* and *MYD88* (in 10% and 29–33% of ABC-DLBCLs, respectively) as well as mutations of *CD79B* (in 12–18% of ABC-DLBCLs), *IRF4*, *EBF1*, and *MALT1*, with mutations of *MYD88* and *CD79B* often coexisting (in >50% of the cases) and basically excluding translocations of *BCL2* and *MYC* [36,45,47, 49,53,63,64,113,146,188]. Genetic alterations in genes such as *PAX5*, *CDKNA2*, *KLHL14*, and *BTG1* have been shown to associate with poor survival whereas alterations in *CREBBP* have been associated with favorable survival in ABC-DLBCL [146].

Though the majority of T-DLBCLs represent the ABC phenotype, the genetic features of T-DLBCL seem to differ from other common ABC-DLBCL entities with some unique combinations of genetic alterations shared with PCNSL [74,103]. In T-DLBCL, *MYD88* mutations have been detected in 68–82% of the cases and *CD79B* mutations in 19–44% of the cases, with 94–100% of *CD79B* mutations occurring concurrently with *MYD88* alterations [74,188,195,196]. Amplifications of NF-κB inhibitor Zeta (*NFKBIZ*) have been described in

44% of T-DLBCL cases, leading to increased expression of inhibitor of nuclear factor kappaB Zeta (IκB-ζ), another essential NF-κB pathway-activating protein in ABC-DLBCL [74,197,198]. Loss of *CDKN2A* has been shown to be significantly more common in T-DLBCL/PCNSL than in nodal ABC-DLBCL (71% vs. 34.5%, respectively) with increased number of total copy number alterations indicating genetic instability [74].

Significantly higher load of IgVH SHMs has been reported in T-DLBCL compared to nodal ABC-DLBCL [103]. Upregulation/rearrangements of forkhead box P1 (*FOXP1*), an oncogene suppressing B-cell apoptosis, and high expression of its protein FoxP1 as well as choline transporters-like protein 1 (CTL1), involved in RNA processing, have been detected in T-DLBCL [103,199-202]. Mutations of *MALT1* have been reported in at least one T-DLBCL case [203]. Additionally, frequent copy number alterations and translocations of 9p24.1 (coding PD-L1/PD-L2) as well as more common loss of *HLA* genes have been identified in both T-DLBCL and PCNSL compared to nodal ABC-DLBCL [74,91,92,199,204-206].

Several distinct genes have been reported to be differentially expressed in T-DLBCL compared to other extranodal and nodal ABC-DLBCLs [103]. In line with mutational findings described earlier, increased mRNA expression levels of genes such as *CARD11* and *MYD88* have been detected in T-DLBCL [103,196]. Expression levels of other genes involved in signaling pathways, cell growth regulation, DNA repair, and cellular metabolism have been reported to be either higher or lower in T-DLBCL compared to other extranodal ABC-DLBCLs whereas expression levels of *HLA* genes as well as genes encoding various extracellular matrix proteins, adhesion molecules, and molecules involved in cytoskeletal reorganization have been described to be lower in T-DLBCL [103,207,208]. As a conclusion, the genetic findings in T-DLBCL in general highlight significant NF-κB/TLR-mediated signaling, often with concurrent BCR pathway activation.

2.4 Tumor microenvironment (TME)

The TME of B-cell lymphomas consists of several different immune cell subpopulations, including TILs, TAMs, NK cells, and DCs, in addition to blood vessels and extracellular matrix [38]. The innate aim of tumor-infiltrating immune cells is to induce anti-tumor immune response. Tumor cells, however, have several mechanisms to escape this immune response, giving the tumors a growth advantage [20,209]. Increasing evidence has demonstrated host immune response predicting the outcome of lymphoma patients. In indolent lymphomas, the prognostic value of TILs was recognized already in the 1980s [210,211]. Thereafter, several studies have been published on host T-cell immune response, TILs, and TAMs in aggressive lymphomas, and the possible mechanisms behind tumor immune escape are beginning to be uncovered [38,212-223]. Since this thesis is addressed on T-DLBCL, the subsequent literature review on TME is mainly focused on DLBCL and T-DLBCL.

2.4.1 Tumor-infiltrating lymphocytes (TILs)

TILs have a dominant role in developing host immune response against tumor cells [209,224-226]. To initiate the cellular immune response, MHC proteins are needed for antigen presentation as MHC proteins present antigens to T-cells through T-cell receptor (TCR) [227]. MHC class I proteins HLA-A, HLA-B, and HLA-C are coded by the corresponding *HLA* class I genes (*HLA-A, HLA-B,* and *HLA-C*, respectively), are expressed on all nucleated cells, and are responsible for antigen presentation to $CD8^+$ T-cells [227]. The regulation of MHC class II expression is more complicated, and several HLA class II protein coding *HLA* class II genes such as *HLA-DQA1, HLA-DRB1, HLA-DPA1*, and *HLA-DQB1* have been recognized [228]MHC class II, consisting of HLA class II proteins, is expressed only on APCs such as DCs, B-cells, monocytes, and macrophages and present antigens to $CD4^+$ T-cells [227].

TILs can be divided into $CD4^+$ T-cells (mainly T helper cells (Th cells) and regulatory T-cells (T_{reg}s)) and $CD8^+$ T-cells (mainly cytotoxic T-lymphocytes (CTLs)). Activation of both $CD4^+$ and $CD8^+$ T-cells occurs mainly in lymph nodes and requires proper antigen presentation by DCs via MHC class I and MHC class II, with an additional co-stimulatory signal mediated by B7-CD28 interaction [225,229]. Subsequently, the activated T-cells migrate to their target tissue in order to perform their effector functions [229]. The mechanisms of T-cell activation, migration, and function are complex, and the main characteristics of these mechanisms are described in more detail below. The most important features of T-cell activation are also presented in Figure 4.

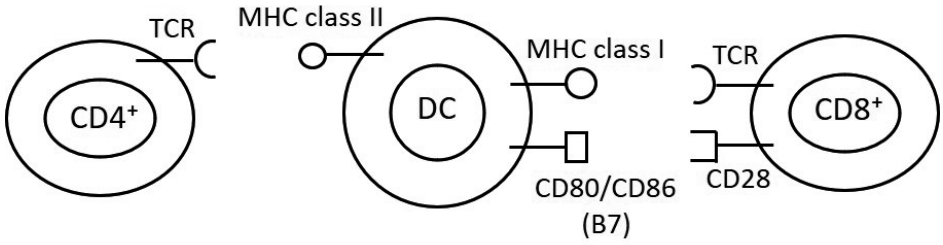

Figure 4. The activation of $CD4^+$ T-cells and $CD8^+$ CTLs. The activation of both $CD4^+$ T-cells as well as $CD8^+$ CTLs requires antigen presentation by DCs via MHC class I and MHC class II proteins that present antigens to T-cells through TCR, as well as an additional co-stimulatory signal through B7-CD28 interaction. Adjusted from Borst et al. 2018 [231].

2.4.1.1 CD4+ T-cells, T helper cells

CD4+ T-cells represent a heterogeneous group of immune cells with a wide spectrum of distinct functions including antigen recognition, immune response activation, as well as immune response downregulation [232]. In the TME, CD4+ T-cells can regulate tumor cell function directly by eliminating tumor cells or indirectly by modulating the TME [231,233].

The division of CD4+ T-cells into different subtypes has been acknowledged since the 1980s when two distinct subtypes of CD4+ T-cells with different cytokine production were recognized [234]. Thereafter, additional evidence has demonstrated more specific subtypes that can be either distinguished into different mature cell lineages already in the thymus, such as T_{reg}s, or can later differentiate from naïve CD4+ T-cells, such as Th cells (Th1, Th2, Th3, and Th17) and induced regulatory T-cells (iT_{reg}s) [232,235-238]. The differentiation of CD4+ TILs is regulated by cytokines and the migration to TME by molecules like L-selectin (CD62L), Figure 5 [239]. In the next chapters, the activation and differentiation of distinct CD4+ TILs, especially Th1 cells, are reviewed.

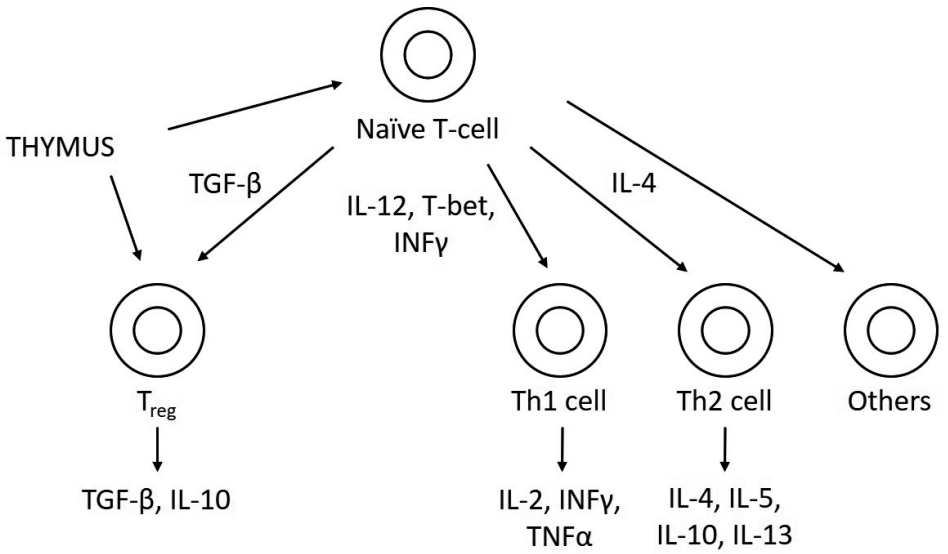

Figure 5. Differentiation of the main CD4+ T-cell subpopulations. CD4+ T-cell subtypes can be distinguished into different mature cell lineages already in the thymus or can later differentiate from naïve CD4+ T-cells. The differentiation of distinct CD4+ T-cell subpopulations, with distinct cytokine production, is regulated by cytokines as shown in the figure.

Adjusted from Kennedy et al. 2008 [233].

Th cells differentiate from naïve $CD4^+$ T-cells and are essential in adaptive immune response. They provide help for both B-cells and T-cells and assist in activating and promoting DC maturation and function [232,233]. In the TME, Th cells also promote the priming and activity as well as effector and memory functions of CTLs (see section 2.4.1.3 for $CD8^+$ T-cell activation and function) [231].

The first recognized Th cell subtypes were Th1 and Th2 cells, with additional other subtypes with different cytokine production and distinct functions having later been described [232,234]. The differentiation to a specific Th cell subtype is induced and regulated by cytokines [240]: T-box expressed in T-cells (T-bet), interferon gamma (INFγ), and interleukin 12 (IL-12), e.g. regulating the differentiation and survival of Th1 cells, whereas IL-4 induces T-cell differentiation towards Th2 cells [232,241-243]. Th1 cells are essential in cell-mediated immune response against intracellular pathogens and have a crucial role in effective anti-tumor immunity while Th2 cells drive host defense against extracellular parasites and suppress the differentiation of Th1 cells and the function of DCs [232,234,237,244,245]. Since Th1 cells are the ones mainly studied in this thesis, the subsequent literature review is focused on Th1 cells.

The primary inducer of Th1 differentiation is IL-12 produced mainly by APCs and mediated by factors such as signal transducer and activator of transcription 4 (Stat4) [246,247]. The functions of Th1 cells are mediated through the production of cytokines such as INFγ, IL-2, and tumor necrosis factor beta (TNFβ) [232,241]. INFγ produced by Th1 cells induces macrophage activity against microbes, and the activated M1 macrophages produce cytokines such as IL-12, further inducing a positive feedback loop of Th1 differentiation [232,248]. INFγ also induce IL-12 receptor expression on antigen-activated Th cells, inhibiting their differentiation towards Th2 cells [249,250]. Additionally, INFγ produced by Th1 cells induces the differentiation of short-lived effector $CD8^+$ T-cells, discussed in more detail in section 2.4.1.3 [243,251].

Although beneficial in infections, a deregulated Th1 response against self-antigens can lead to autoimmune disease, and several regulatory mechanisms for the dampening of Th1 response have been recognized [252]. In addition to self-regulation by IL-10 produced by highly activated Th1 cells, other immune cells, such as T_{reg}s, can downregulate the activity of Th1 cells [235,253,254]. T_{reg}s, being responsible for the regulation of immune response and self-tolerance, are essential for proper regulation of Th1-mediated adaptive immunity, and loss of T_{reg}s has been demonstrated to lead to uncontrolled Th1 responses and aggressive autoimmune disease [235,252,255]. As T_{reg}s are a heterogeneous and versatile group of immune cells, it has been demonstrated that, during strong Th1 response, T_{reg}s can differentiate into a subgroup of T_{reg}s that express both Forkhead box P3 (FoxP3) and T-bet and are optimized for the suppression of Th1 cells [243,252,256]. Differentiation and function of T_{reg}s are further discussed in section 2.4.1.2.

T-bet is a transcription factor crucial for Th1 differentiation and commonly recognized as a Th1 cell marker [241,242]. In addition to Th1 cells and T_{reg}s, T-bet can be expressed by thymocytes, DCs, NK cells, and innate lymphoid cells [241-243]. The expression of T-bet can be induced by TCR, IFNγ-Stat1, or IL-12-Stat4 signaling pathways, with also other mechanism of T-bet expression upregulation having been suggested, such as autoactivation of T-bet coding *Txb21* [241,243,257-259]. Similar to INFγ, T-bet can promote Th1 differentiation as well as suppress the development of other Th cell lineages [243]. When co-expressed with FoxP3 in

T_{reg}s, T-bet can, however, have also adverse effects with Th1 cell suppressing functions [252]. In Th1 cells, T-bet promotes the production of INFγ, introducing yet another self-directing loop of Th1 cell differentiation, independent of IL-12 [242].

2.4.1.2 Regulatory T-cells

T_{reg}s are involved in regulating immune response and self-tolerance [235]. They were first recognized in the 1990s when CD25 expressing $CD4^+$ T-cells were shown to suppress immune response and maintain self-tolerance [260]. Later, it has been described that T_{reg}s can be divided into $CD4^+$ "natural" T_{reg}s (nT_{reg}s) produced by normal thymus and iT_{reg}s differentiated from naïve $CD4^+$ T-cells [232,235]. These can be further divided into different subgroups with distinct functions, and also additional other subgroups of T-cells, such as a subpopulation of $CD8^+$ T-cells have been shown to have regulatory functions [236,256]. Furthermore, T_{reg}s can emerge to their target tissue through different paths including migration from circulating blood or the lymphatic system, differentiation resulting from DC activation, and transforming growth factor beta (TGF-β) induced differentiation from naïve $CD4^+$ T-cells into T_{reg}s [229,252,256,261-266]. Altogether, several distinct subtypes of T_{reg}s have been described and since this thesis mainly studies $CD4^+FoxP3^+$ T_{reg}s, the following literature review is focused on describing the function and regulation of this specific subtype [236,256].

FoxP3 is a transcription factor expressed in $CD4^+$ T_{reg}s and tumor cells [236,266,267]. It is known as the key regulatory gene in the development of nT_{reg}s and is a commonly recognized Treg marker [266-268]. The expression of FoxP3 is essential in maintaining the suppressive activity of T_{reg}s, and in case of a strong Th1 response, INFγ-mediated additional expression of T-bet is required for the proper migration and maintaining of the functional capability of T_{reg}s [243,252,269]. The expression of FoxP3 in T_{reg}s can be regulated by different signaling pathways, such as TCR and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR), epidermal growth factor receptor (EGFR)/glycogen synthase kinase 3 beta (GSK-3β)/FoxP3, hypoxia-inducible factor-1 alpha (HIF1A), and others [267,270-276]. In addition, TGF-β expressed by another T_{reg} subtype, Th3 cell, can induce the differentiation of naïve $CD4^+$ cells into $CD4^+FoxP3^+$ iT_{reg}s through IL-2-Stat5 pathway [263-265,277,278].

$CD4^+FoxP3^+$ T_{reg}s suppress immune response against self-antigens and tumors and have been demonstrated to suppress the function of other $CD4^+$ TILs as well as other anti-tumor immune response cells such as NK cells, macrophages, DCs, and B-cells [212,232,236,256,267,279]. The immune suppressive functions of T_{reg}s can occur through several different mechanisms including the secretion of inhibitory cytokines and other molecules such as TGF-β, IL-10, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3), and granzyme B (GrB) [279]. Additionally, IL-10 produced by T_{reg}s may upregulate the expression of inhibitory programmed cell death ligand 1 (PD-L1) on DCs, leading to CTL exhaustion (see section 2.4.1.4 for T-cell exhaustion) [280].

2.4.1.3 CD8+ T-cells

CD8+ TILs have a dominant role in cell-mediated anti-tumor immune response [229]. In the TME, CD8+ CTLs detect intracellular antigens presented by MHC I molecules on all tumor cell types and are responsible for killing the tumor cells by granule exocytosis and Fas ligand (FasL)-mediated apoptosis induction [229,281]. Additionally, CTLs interact with other immune cells of the TME by producing cytokines such as INFγ and tumor necrosis factor alpha (TNFα) [281].

The optimal function of CD8+ TILs requires their attachment to endothelial cells, followed by their proper migration and differentiation, and is regulated by several different mechanisms [229,239,282-284]. CD8+ TILs are recruited to tumor site from peripheral blood by cytokines such as IL-12, chemokine (C-C motif) ligands 4 and 5 (CCL4 and CCL5), and chemokine (C-X-C motif) ligands 9, 10, and 11 (CXCL9, CXCL10, and CXCL11) [229]. The migration of CD8+ TILs to the TME is regulated by molecules like CD62L, enabling the attachment of CD8+ TILs to endothelial cells [239]. Myeloid-derived suppressor cells (MDSCs) can, in contrast, downregulate the migration by mechanisms including the reduction of IL-12 secretion and the suppression of CD62L expression in both CD4+ and CD8+ T-cells [239,285].

Traditionally, CD8+ T-cells have been divided into effector T-cells and memory T-cells: effector T-cells being the ones active in acute infections and vaccinations with the requirement of antigen presentation by DCs and the co-stimulatory signal through the B7-CD28 pathway to be activated; memory T-cells being T-cells that can remain after the ongoing antigen presentation and inflammation has ceased and again be re-activated upon re-stimulation [286-289]. In the TME, with continuous antigen exposure, the evolution of CD8+ T-cells is different and the majority of effector cytotoxic T-cells have been shown to obtain a state of exhaustion instead of converting into memory T-cells (see section 2.4.1.4 for T-cell exhaustion) [288,290]. In previous literature, also Th cells and memory T-cells are considered as effector T-cells alongside with CTLs [229,232]. For clarification, in this literature review, the term CTL is used to specify a subset of CD8+ TILs with cytotoxic activity instead of using the term effector cytotoxic T-cell. T-cell exhaustion is reviewed in more detail later in section 2.4.1.4 whereas this chapter describes the regulation of CD8+ TIL activation and function.

CTLs are a subset of differentiated CD8+ effector T-cells that are essential in immune response against tumor cells. As described earlier in section 2.4.1, the activation of CTLs, so-called 'T-cell priming', requires both antigen presentation and a co-stimulatory signal from DCs, Figure 4. Additionally, CTL-mediated anti-tumor immune control is assisted by essential help from Th cells, mediated by a specific gene program that downregulates coinhibitory receptors such as PD-1 and increases the motility and migration capacities of CTLs by inducing the expression of chemokine receptors and metalloprotease activity [251]. The activation of Th cells also assists DCs in both optimizing antigen presentation to CTLs as well as in promoting CTL clonal expansion and differentiation by releasing cytokines and delivering co-stimulatory signals [231,251,291].

The help from CD4+ T-cells is largely mediated via CD70–CD27 interaction although several other mechanisms and pathways also exist, Figure 6 [251]. The simultaneous activation of CD4+ and CD8+ T-cells leads to expression of CD40L on activated CD4+ Th cells [292]. CD40L interacts with CD40 on DCs, further activating the upregulation of CD80, CD86, and CD70

in DCs and triggering the co-stimulatory receptors CD27 and CD28 on $CD8^+$ CTLs [251,293-295]. The subsequent CD70–CD27 interaction leads to activation of CTLs. It has been discussed whether CD40L can also directly stimulate CTL differentiation by binding to CD40 in CTLs, but the reports on this have been inconsistent [291,296-299]. Additionally, interactions between DCs and NK cells seem to be able to substitute Th-mediated help by stimulating Th1- and Th2-related cytokine release from NK cells, and lack of help from $CD4^+$ Th cells has been associated with lower expression of killer cell lectin-like receptor G1 (KLRG1), CD127, INFγ, GrB, and T-bet [251,300,301].

Stimulation by extracellular cytokines such as IL-2, IL-7, IL-15, and IL-21 can promote the activation of CTLs, and as with $CD4^+$ T-cells, CTLs can also induce their own activity by secreting cytokines such as IL-12 and INFγ, promoting the differentiation of $CD8^+$ T-cells into effector CTLs [229,231]. Additionally, CTLs express several co-stimulatory and inhibitory receptors that can further modify their activity [302-304].

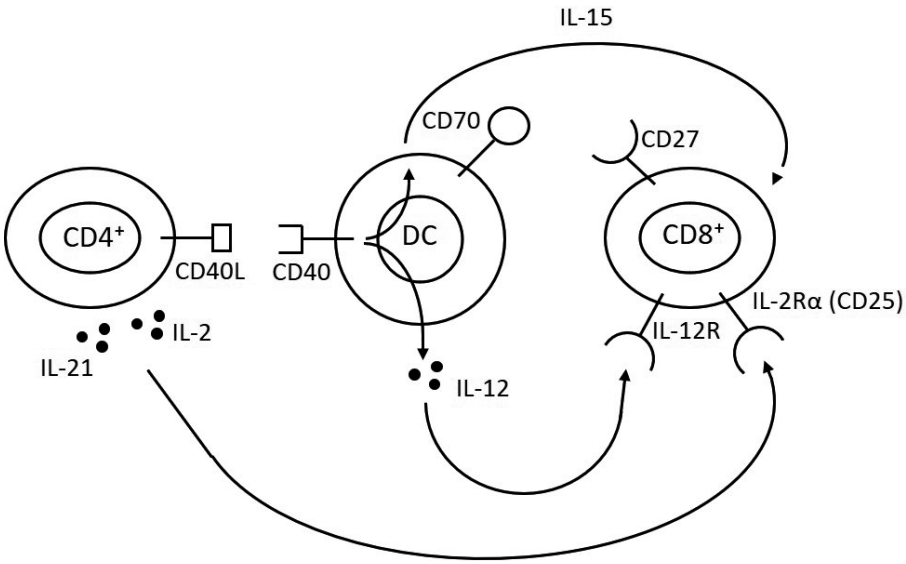

Figure 6. The activation of CTLs. The simultaneous activation of $CD4^+$ and $CD8^+$ T-cells leads to the expression of CD40L on activated $CD4^+$ Th cells. CD40L interacts with CD40 on DCs, and further activates the upregulation of CD70 in DCs and triggers the co-stimulatory receptor CD27 on $CD8^+$ CTLs. Cytokines such as IL-2, IL-12, IL-15, and IL-21 can further promote the activation and differentiation of CTLs.

Adjusted from Borst et al. 2018 [231].

In the TME, activated CTLs kill tumor cells by granule exocytosis and FasL-mediated apoptosis. The granule exocytosis pathway is activated by granzymes A and B (GrA and GrB) released by CTLs [305-308]. The released granzymes enter tumor cells and lead to their death by several distinct mechanism, including the induction of DNA damage, loss of cell membrane integrity, and destruction of nuclear envelope [305,306,309]. The FasL pathway of apoptosis is mediated by CTL activation of FasL, leading to subsequent cytochrome c release in the target cells, activation of caspases, and tumor cell apoptosis [309,310]. In addition, cytokines such as IL-2, INFγ, and TNFα released by CTLs can induce the anti-tumor activity of other immune cells such as M1 macrophages as well as their cytotoxicity against tumor cells (discussed in more detail in section 2.4.2) [311-313].

2.4.1.4 T-cell exhaustion

The primary role of TILs is to induce anti-tumor response. The TME, however, serves a distinct milieu for T-cells to function as the antigen exposure is prolonged and antigen removal unsuccessful, leading to a state of exhaustion that was first recognized in viral infections and has thereafter been described in tumors [314-319]. In recent years, knowledge on tumor biology and T-cell exhaustion has increased and led to major advancements in the medical field as immune checkpoint inhibitors are already commonly used in the treatment of many malignancies including lymphomas. In this chapter, the mechanisms of T-cell exhaustion are selectively reviewed, focusing on the interactions between the tumor and the host immune cells, inhibitory receptors, cytokine milieu, and other inflammatory cells, leaving out a thorough description of the metabolic conditions and other environmental issues in the TME also involved in T-cell exhaustion [230].

Tumor cells have several mechanisms of escaping the T-cell-mediated anti-tumor immune response. The mechanisms include direct interactions between tumor and host immune cells, altered expression of surface molecules, and recruitment of immunosuppressive cells that downregulate T-cell activation [38,223,225,230,320-324]. In CTLs, T-cell exhaustion leads to upregulation of inhibitory receptors, decreased production of effective cytokines, and reduced cytotoxic activity and proliferation [230]. The activation of naïve T-lymphocytes against tumor cells is also impaired by inadequate tumor-specific antigen presentation and by downregulation of CTL activation, mediated by other host immune cells [230].

The interactions between tumor cells and CTLs is altered by different mechanisms leading to impaired CTL activation and function as well as induced apoptosis of CTLs [230,325]. Tumor cells express molecules such as programmed death cell ligand 1 (PD-L1) and FasL [310,321]. The expression of PD-L1 has been associated with decreased infiltration of CTLs and has been suggested to induce CTL apoptosis [230,320]. Nevertheless, further studies have shown PD-L1 expression associating with immune escape and T-cell non-responsiveness rather than apoptosis induction [209,288]. The function of FasL depends on the microenvironment [310,324]. It can both promote anti-inflammatory and anti-tumor responses and induce apoptosis of CTLs [310,324,325].

Exhausted phenotype T-cells express inhibitory receptors such as PD-1, LAG-3, T-cell immunoglobulin and mucin domain containing-3 (TIM-3), and CTLA-4 [230,288,317,318,326-328]. In normal conditions, the initial purpose of these inhibitory receptors is to downregulate

excessive immune response, autoimmune activity, and tissue damage. In the TME, however, the activation of inhibitory pathways leads to impaired immune response, T-cell exhaustion, further induction of the expression of these inhibitory receptors, and gradual loss of the ability of CTLs to produce cytokines such as IL-2, TNFα, INFγ, and GrB [230]. Both the overexpression of inhibitory receptors and decreased cytokine production have been shown to increase in line with prolonged antigen exposure [314]. The functions of PD-1, LAG-3, and TIM-3 are subsequently briefly reviewed. The activation and function of CTLA-4 and other inhibitory receptors are not further discussed in this review as they were not studied in this thesis.

PD-1 was first discovered in the 1990s as a gene upregulated in T-cell hybridoma undergoing cell death [329]. It is currently known as an inhibitory receptor expressed predominantly by TILs and can also be expressed by B-cells and other immune cells [321,330]. Its ligands PD-L1 and PD-L2 can be expressed by tumor cells as well as other cells in the TME [331-333]. In normal conditions, the expression of PD-1 is induced following TCR engagement, and is eventually reduced as the antigen exposure ceases [230,321]. In tumors, antigen exposure is prolonged, leading to sustained PD-1 expression on TILs, and the PD-1–PD-L1 interaction between CTLs and tumor cells has been shown to be one of the hallmark pathways in T-cell exhaustion [209,321]. The mechanisms of PD-1-mediated T-cell downregulation are not fully understood but alterations such as modulation of TCR signaling and PI3K/AKT/mTOR pathway have been suggested [230]. On the other hand, the effect of PD-1-targeted therapies has been shown to be CD28-dependent, demonstrating that PD-1-mediated T-cell inhibition mainly occurs through inactivation of the co-stimulatory CD28-B7 signaling [334-336].

LAG-3 is an MHC II ligand expressed on activated and exhausted T-cells, B-cells, and DCs [230,337-339]. It enables the maintenance of tolerance to self and tumor antigens by directly suppressing the activity of $CD8^+$ T-cells [340]. It has been reported to function synergistically with other inhibitory receptors such as PD-1 in promoting T-cell exhaustion [341,342]. Additionally, LAG-3 suppresses $CD4^+$ T-cell expansion, promotes the function of T_{reg}s, and modulates the activity of DCs [337,338,343-345]. It has been suggested as a marker for T_{reg}s and their activity [344]. The LAG-3-induced modulation of DC function is mediated by upregulation of co-stimulatory molecules and by production of IL-12 and TNFα, leading to DC maturation and activation [345].

TIM-3 is an immunoregulatory checkpoint receptor expressed by most lymphocyte subtypes including exhausted T-cells and NK cells and is regulated similarly to PD-1 [230,346,347]. The expression of TIM-3 leads to inadequate T-cell function and decreased production of cytokines such as IL-2, TNF, and INFγ [348-350]. On TILs, the expression of TIM-3 seems to enhance T-cell exhaustion when co-expressed with PD-1 [348].

T-cell exhaustion can be mediated by cytokines produced by tumor cells, TAMs, or T_{reg}s [230,351]. Cytokines such as TGF-β and IL-10 downregulate T-cell activity and modulate the activity of other immune cells while IL-12 has been reported to induce T-cell exhaustion by the induction of LAG-3 and TIM-3 [230,352,353].

Interactions between CTLs and other immune cells are complex, and CTLs can both antagonize and support the activity of T_{reg}s [313]. As described earlier in section 2.4.1.3, $CD4^+$ Th cells are crucial in CTL activation and downregulation of T-cell exhaustion [231]. $CD8^+$ T-cells primed in the absence of $CD4^+$ Th cells have been shown to express significantly higher levels of co-inhibitory receptors such as PD-1, BTLA, LAG-3, and CD200 receptor (CD200R) (and

its ligand CD200) and have significantly less cytotoxic activity than $CD8^+$ T-cells primed with help from $CD4^+$ Th cells [251]. The activity of $CD8^+$ T-cells primed without help from $CD4^+$ Th cells could, however, be improved with specific anti-PD-1, anti-BTLA, and anti-LAG-3 mAbs, indicating a reversible inhibition of these receptors [251].

2.4.2 Tumor-associated macrophages (TAMs)

Macrophages are an essential part of both innate and adaptive immune systems with their capability of phagocytosis and the ability of inducing T-cell activation and recruitment. Classically, macrophages have been divided into two subtypes which can roughly be described as tumor suppressive, classically activated M1 macrophages and pro-tumor, alternatively activated M2 macrophages [354-356]. Nonetheless, macrophages are a heterogeneous and flexible group of cells that can modify their phenotype according to the surrounding microenvironment [357-361]. Growth factors, cytokines, and chemokines drive the differentiation of macrophages, and especially the alternatively activated M2 macrophages can be further divided into different subtypes with distinct functions [355,362-367].

Macrophages are phagocytic cells, present in basically all tissues, and have a role in immune responses as well as in maintaining metabolic homeostasis [362]. They derive from circulating peripheral-blood mononuclear cells that develop from common myeloid progenitor cells in the bone marrow [363,368]. The common myeloid progenitor cells (granulocyte/macrophage colony-forming units) undergo differentiation into monoblasts, pro-myelocytes, and finally monocytes before leaving the bone marrow and entering the peripheral blood [363]. Monocytes from peripheral blood migrate to tissues, developing further into macrophages or DCs [363,369]. The differentiation is induced by cytokines such as IL-10 which promotes the differentiation towards macrophages and inhibits the differentiation towards DCs [370,371]. It has been suggested that monocytes could mature and differentiate also in the bloodstream, and the point of recruitment to the tissue during this maturation could define their function in the tissue [362]. Additionally, populations of mature macrophages in the tissue may also arise through proliferation of tissue-resident colony-forming cells [362,363].

The role of macrophages in maintaining metabolic homeostasis includes removal of cellular debris generated by either tissue remodeling, necrosis caused by stress or trauma, or cells that have undergone apoptosis [362]. Macrophages recycle the material to be reused by the host, and the material phagocytosed by macrophages can alter their physiology by causing changes to their expression of surface molecules as well as the production of cytokines and other inflammatory mediators such as TGF-β, TNFα, and ILs [362,372-374]. The endogenous danger signals (proteins, histones, DNA and other nucleotides, components of the extracellular matrix) phagocytosed along with cellular debris are detected by macrophages through receptors such as TLR, intracellular pattern-recognition receptors, and interleukin-1 receptor (IL-1R), further inducing macrophage activity [375-377]. The induced macrophage activation can either initiate, enhance, or inhibit inflammation through various signaling pathways including the NF-κB pathway [376,377]. This macrophage activation is independent of adaptive immune response, indicating a significant role of macrophages in detecting possible danger and participating in both innate and adaptive immune responses [372,376]. Additionally, the phenotype and physiology of macrophages can be altered by signals from both innate and

adaptive immune responses that can either enhance or downregulate macrophage activity [362]. The most important features of macrophage differentiation and activation are presented in Figure 7.

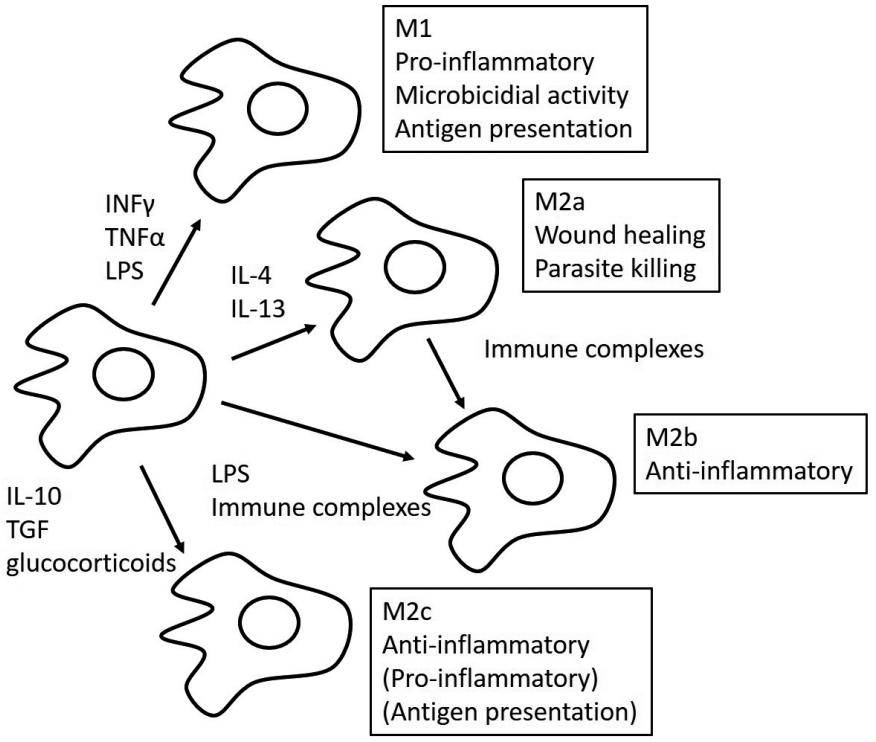

Figure 7. Macrophage differentiation. Macrophage activation can either initiate, enhance, or inhibit inflammation, and macrophages can be divided into different subtypes with alternative functions. The phenotype and physiology of macrophages can, however, be altered by signals from both innate and adaptive immune responses. The factors inducing the activation of different macrophage subtypes with distinct functions are described in the figure.

Adjusted from Gordon et al. 2005 [363].

M1 macrophages are produced either during cell-mediated immune response or in response to innate stimuli from stress reaction or viral infection [362]. They are defined as classically activated phenotype with potent microbicidal properties and capability to produce IL-12 and promote IL-12-mediated Th1 cell response (Th1 cells are reviewed more closely in section 2.4.1.1) [366,367]. They are also highly capable of functioning as APCs [378]. The activation of M1 macrophages is induced by factors such as INFγ, TNFα, and lipopolysaccha-

rides (LPS) through several possible mechanisms [366,367,378]. Innate immune cells such as NK cells can induce macrophages in their killing ability and the production of pro-inflammatory cytokines by producing INFγ as an early response to infections, whereas more sustained production of INFγ by the adaptive immune response-mediating Th1 cells maintains M1 activity and enables a stable host defense against intracellular pathogens [362]. Since TAMs are considered to mainly represent the alternatively activated phenotype, the other possible mechanisms of M1 activation are not described here in more detail, and the subsequent literature review is focused on the M2 phenotype [362].

Alternatively activated M2 macrophages can be further divided into different subgroups with distinct activating stimuli and functions [366,367]. Exposure to IL-4 or IL-13 activates macrophage differentiation towards a distinct phenotype that can be named M2a; exposure to immune complexes together with pro-inflammatory stimuli such as LPS differentiation towards another phenotype, M2b; and exposure to IL-10, TGF-β, or glucocorticoids towards yet another different phenotype, named M2c; Figure 7 [366,367]. Additionally, IL-6 and leucine-rich repeat-containing G-protein-coupled receptor (Lgr4) mediated signaling have been demonstrated to be involved in M2-like differentiation of TAMs [360,379].

The differentiation of so-called wound healing M2a macrophages is considered to be mainly induced by the adaptive immune response [362]. Tissue injury leads to activation of IL-4- and IL-13-mediated Th2 response and further activation of M2a macrophages [380,381]. M2a macrophages secrete components of extracellular matrix, suppress the clonal expansion of neighboring lymphocytes, and are less efficient at producing pro-inflammatory cytokines and at killing intracellular pathogens than M1 macrophages [362]. They also lack the ability to induce T-cell activation and have been shown to present with decreased production of both Th1 cell-inducing IL-12 and immunosuppressive IL-10 [367].

The presence of immune complexes together with pro-inflammatory stimuli such as LPS can induce macrophage differentiation towards potent anti-inflammatory M2b macrophages [367,382]. The IL-4 primed M2a macrophages can also convert into M2b-like phenotype in the presence of immune complexes [367]. M2b macrophages present with altered cytokine production such as increased production of IL-10 and decreased production of IL-12 [367,382]. These M2b macrophages can, however, induce the activation of IL-4-, IL-10-, and IL-13-producing Th2 cells [232,367].

In stress reactions, exposure to glucocorticoids can alter macrophage activity by affecting Th1/Th2 balance through suppressing the production of Th1 cell inducing IL-12 and shifting the balance of adaptive immune response towards Th2 cell activation [383-386]. The phagocytotic activity of apoptotic cells of macrophages is retained or increased in response to glucocorticoids, leading to their increased secretion of regulatory molecules such as TGF-β [373,387]. Additionally, glucocorticoids suppress NF-κB pathway activation and the production of pro-inflammatory cytokines such as IL-1, IL-6, and TNF, whereas the production of anti-inflammatory cytokines such as IL-10 is increased in response to glucocorticoids [384,388,389]. Exposure to regulatory factors such as TGF-β, IL-10, and glucocorticoids induces macrophage differentiation towards regulatory M2c macrophages [362,366]. Analogous to M2b macrophages, M2c macrophages inhibit inflammation by producing high levels of immunosuppressive IL-10 and by downregulating the production of IL-12 [362,363,390]. They might, however, also retain their ability to produce pro-inflammatory cytokines and present antigens to T-cells [362].

TAMs play a crucial role in the TME. They collaborate with B-cells and T-cells through direct cell-to-cell interactions as well as through different cytokines, chemokines etc. It seems that TAMs may be able to have alternative roles in the tumor depending on their phenotype, the TME, and the phase of tumor evolution [351,358,362,378,391-401].

TAMs are often considered to mainly represent M2-like phenotype [351,357]. They have been associated with induced tumor angiogenesis and poor prognosis [358,402-404]. Some TAMs have been described to share common characteristics with regulatory M2c macrophages, and lower content of TAMs has been reported to have no effect on tumor evolution or to be beneficial to the host, supporting the assumption of M2c phenotype [358,405,406]. Induced TAM infiltration has, however, been associated with both increased tumorigenicity and delayed tumor growth while blocking of monocyte recruitment has been demonstrated to promote tumor growth [396,397,407,408].

It seems that TAMs are a versatile population of tumor-infiltrating immune cells, and it has been described that by changes in the TME, TAMs could convert their phenotype from M1 towards M2c-like during tumor evolution [362,393,399]. TAMs have been shown to secrete high levels of pro-inflammatory TNFα, to eliminate transformed or pre-transformed cells, and to be essential in tumor rejection, thereby perhaps being involved in the early tumor evolution with M1-like tumor suppressive functions [378,395,409-417]. Later on, M2c-like TAMs can produce high levels of IL-10, little or no IL-12, and decreased levels of TNFα [351,378,401,409]. They suppress the activity of other APCs and have several other pro-tumor functions including decreased tumoricidal capacity, downregulation of neighboring TAMs, and the already mentioned induction of angiogenesis [362,378,402-404,418].

Altogether, TAMs are a highly flexible immune cell population with changing roles depending on their microenvironment. The interactions between TAMs, tumor cells, and other host immune cells are discussed further in section 2.4.3.

2.4.3 Host-related factors and immune escape

The role of host immunity, other host-related factors and immune escape is becoming increasingly acknowledged in many tumors, including lymphoid malignancies [18,38,230,378,419,420]. Lymphomas can be seen as a dysfunction of the immune system *per se*, as they develop from immune cells: B-cells, T-cells, or NK cells. The additional changes in the TME lead to a very delicate and complex system with numerous different immune cells, cytokines, chemokines, receptors, ligands, pathways as well as extracellular matrix and exosomes involved in the tumor pathogenesis and evolution [18]. The function and role of B-cells, macrophages, and T-cells have been reviewed more thoroughly in sections 2.1, 2.4.1, and 2.4.2, whereas this section is focused on summarizing the changes in the TME and the mechanisms by which the tumor can alter the functions of the host immune system, further enabling its escape from host immunity.

CTLs are a T-cell subpopulation essential in cell-mediated host immune defense against tumor [229,230]. As described earlier in section 2.4.1, the activation and function of CTLs is highly complicated and regulated by numerous cytokines, chemokines, and cell-to-cell interactions. The proper activation and function of CTLs require antigen presentation with co-stimulatory signals from DCs as well as help from Th cells or NK cells [251,300]. Conversely, cancer-associated fibroblasts (CAFs), M2-like macrophages, and T_{reg}s have immunosuppressive

functions and can downregulate the CTL-mediated anti-tumor immune response [281]. Additionally, in the TME, continuous antigen exposure can drive CTLs into a state of exhaustion with reduced cytotoxic activity and proliferation [230].

CTLs release factors such as GrA, GrB, TNFα, and INFγ to induce their cytotoxicity against tumor cells [281,352]. The same cytokines can induce the activation of other immune response cells with anti-tumor activity, such as M1 macrophages [366]. The production of these tumor suppressive factors by CTLs can be reduced by immunosuppressive cytokines such as TGF-β produced by the tumor cells, T_{reg}s, and M2-like TAMs [230,264,352,366,367,373]. In addition to tumor progression induction, TGF-β can induce macrophage differentiation towards immune-suppressive M2c-like phenotype, promote the immunosuppressive activity of T_{reg}s, and suppress the proliferation of $CD4^+$ and $CD8^+$ T-cells [281,366,367,421,422]. Tumor cells can also induce the suppression of CTL by secreting factors like adenosine [423-425]. Additionally, tumor cells can release other immunosuppressive mediators such as PD-L1 and STAT3 as well as inhibit macrophage polarization towards anti-tumor M1 phenotype by secreting factors like tumor-secreted protein S (Pros1) [281,361,426]. Lymphoma cells can further escape from macrophage-mediated destruction by CD47-mediated activation of signal regulatory protein alpha (SIRPα) [427].

T_{reg}s are immune suppressive cells regulated by several distinct mechanisms of the immune system and the TME [428,429]. They reduce responses of both innate and adaptive immune systems and collaborate with numerous different immune cell subtypes including M2 macrophages and CAFs [281,420,430]. They can suppress the activity of CTLs by releasing TGF-β and other immunosuppressive factors as well as through adenosine-related pathways [281,425,428].

TNFα has originally been considered as a pro-inflammatory cytokine produced by macrophages and CTLs [281,409,410]. It induces the anti-tumor functions of several immune cells, including M1 macrophages and CTLs [281,354]. Recent observations have, however, also revealed possible pro-tumor functions induced by TNFα [281,431]. These observations are still preliminary and require additional studies to be thoroughly understood [281].

INFγ is another pro-inflammatory cytokine produced by NK cells, CTLs, $CD4^+$ T-cells, and macrophages [281,362]. It promotes the activation and differentiation of anti-tumor M1 macrophages, Th1 cells, and $CD8^+$ T-cells, inhibits Th cell differentiation towards Th2 cells, but also induces PD-L1 and STAT3 expression on M1 TAMs and tumor cells, leading to the expression of co-inhibitory receptors such as PD-1 on activated T-cells and further T-cell exhaustion [232,250,281,366].

Macrophages are part of the innate and adaptive immune systems. They can derive into different subtypes with distinct functions according to their microenvironment [351,361,378,379,432-434]. TAMs have been demonstrated to have alternative roles depending on tumor phase and TME-related factors and seem to be able to convert from tumor suppressive M1-like macrophages into pro-tumor M2-like macrophages and vice versa [358,378,435-438]. Especially in the early stages of tumor evolution, TAMs have been described to have M1-like tumor suppressive functions such as elimination of tumor cells and secretion of pro-inflammatory cytokines like TNFα [409,410,412-417]. By contrast, TAMs have also been demonstrated to be capable of directly promoting tumor angiogenesis as well as tumor growth and metastasis [378,404,435,439,440].

TAMs have been described with decreased ability to lyse tumor cells [404,441,442]. The phagocytotic activity of TAMs can be inhibited by NF-κB-regulated expression of immune checkpoint molecules such as PD-1 [443,444]. TAMs have been reported to resemble M2 macrophages by their cytotoxicity and the production of cytokines [351,401,442,445]. In addition to over-expression of IL-10, the immunosuppressive phenotype of TAMs has been associated with defective activation of NF-κB [401,409].

TAMs can have several different mechanisms of regulating T-cell activity [404]. As one of the main functions of macrophages is to induce T-cell recruitment and activation, in the TME, this function is disturbed by the poor tumor-associated antigen-presenting capability of TAMs [404]. Additionally, M2-like TAMs produce high levels of Th1 cell-suppressing IL-10 and shift the Th1/Th2 balance towards Th2 cell activation [378]. The IL-10 produced by M2-like TAMs further suppresses the effective Th1-CTL-mediated anti-tumor immune response, but can also decrease T-cell activation by suppressing the function of other APCs such as DCs [370]. Additionally, the M2-like TAM induction and increased IL-10 production induces the activation of T_{reg}s, leading to further suppression of immune response against tumor [370,446].

CD20 targeting rituximab was the first mAb introduced in lymphoma treatment. It has been demonstrated to accomplish its therapeutic effects through the induction of adaptive cellular immune response as both $CD4^+$ and $CD8^+$ T-cells seem to be needed for achieving treatment response [447,448]. Complement activation, NK cells, neutrophils, and macrophages have also been reported to be involved in the Ab-dependent cell-mediated cytotoxicity [449-453]. Interestingly, M2-like macrophages have been shown to phagocytose rituximab-opsonized cells more efficiently than M1 macrophages [454]. Anti-CD47 Ab seems to promote the phagocytosis by synergizing with rituximab [455,456].

Increased knowledge on the complexity of the TME and host-related factors involved in tumor immune escape are revealing new opportunities to interfere with pro-tumor as well as tumor-suppressive factors [18,21,38]. Immunotherapy and especially host T-cell-specific immune response promoting therapies have lately been the main focuses of new therapeutic approaches in many malignancies including lymphomas [231,281,457-461]. A high number of CTLs have been associated with better prognosis of patients, and tumors with high density of CTLs, so-called 'immunologic', 'hot', or 'inflamed' tumors, have been reported to respond better to immunotherapy [281,462-465].

In T-DLBCL, the role of host immunity and immune escape seem to be remarkably important [73]. Copy number losses of *HLA* class I and II leading to decreased expression of MHC I and II expression and limited tumor-antigen presentation to TILs, gains and amplifications of *CD274* and *PDCD1LG2* (coding PD-L1 and PD-L2, respectively) with their increased transcription and protein expression implicating immune escape, as well as T-DLBCLs' location in an immune-privilege site are all hallmarks of T-DLBCL and highlight the significance of TME and host-related factors in this specific lymphoma entity [73,74,91,207,466].

As a conclusion, host-related factors and immune escape are extremely eclectic and still not fully understood. One of the main functions of tumors seems to be reprogramming of the host immune cells in the TME towards immunosuppressive activity. Reduced immunogenicity and further immune evasion can also be caused by loss of cell surface molecules and markers on tumor cells leading to decreased recognition by host immune cells [18]. The overexpression of inhibitory receptors on tumor cells interacting with their counterparts on T-cells can lead

to a state of T-cell exhaustion and impaired T-cell-mediated cytotoxicity. Lymphoma cells can furthermore have additional mechanisms leading to e.g. their escape of macrophage-mediated immune response. Mechanisms involving NK cells, DCs, and other factors in the TME have also been recognized but are left out of this review as they are not in the focus of this thesis.

2.4.4 The prognostic value of TME

The prognostic value of numerous distinct factors of the TME has been studied in several tumors including lymphomas [419,435,448]. Ratios of T-cell and checkpoint molecule gene expression levels have been suggested as prognostic biomarkers in DLBCL, independent of R-IPI and COO, as higher proportions of CD4 and CD8 gene expression levels compared to M2 macrophage and PD-L1 gene expression levels have been reported to correlate with better OS [218]. Furthermore, loss of *HLA* class II gene and protein expressions have been shown to associate with lower numbers of $CD8^+$ TILs and worse OS, independent of IPI, among patients with DLBCL [227]. Conversely, in T-DLBCL, frequent loss of HLA class I and II protein expressions has been reported to associate with higher percentage of CTLs, suggesting that the loss of HLA class I and II protein expressions provides a mechanism of immune escape [207].

Based on flow cytometry, higher content of $CD4^+$ TILs has been demonstrated to predict better OS and better event-free survival/relapse-free survival (EFS/RFS) in patients with DLBCL treated both in rituximab and pre-rituximab eras, independently of IPI and R-IPI [213,216,217]. When studied by IHC in combination with FoxP3 and PD-1, higher content of $CD4^+$, $FoxP3^+$, and $PD\text{-}1^+$ cells associated with better OS in DLBCL [467]. The same study reported a correlation between the number of $CD4^+$ cells and $FoxP3^+$ and $PD\text{-}1^+$ cells but could not localize the expression of FoxP3 or PD-1 to a specific cell type. Another study showed that increased infiltration of $PD\text{-}1^+$ cells had an association with prolonged PFS and OS in DLBCL patients treated with R-CHOP but no association with survival according to the proportion of $PD\text{-}L1^+$ cells could be seen [331,467]. Yet another study, however, reported high PD-L1 positivity of the tumor cells predicting inferior OS and associating with non-GCB phenotype and Epstein-Barr virus positivity in DLBCL [468]. PD-L1 positivity of the TME did not have any association to survival in this study, either [468].

Analyzed with IHC, higher FoxP3 positivity has been associated with longer OS of DLBCL patients, a trend towards longer disease-specific survival (DSS) of GCB phenotype DLBCL patients, and a trend towards shorter DSS of non-GCB phenotype DLBCL patients, with FoxP3 positivity, not, however, remaining as an independent prognostic factor in either of these studies [469,470]. In a study combining flow cytometry, gene expression profiling, and cell culturing, higher amounts of $CD4^+CXCR5^+FoxP3^+$ follicular T_{reg}s (Tf_{reg}s) were reported to associate with limited stage disease and longer complete remission (CR) duration [471]. Based on cell culturing examinations, Tf_{reg}s were suggested to be involved in intratumoral immunity and beneficial in DLBCL [471].

The prognostic influence of TAMs in DLBCL seems controversial [219-222]. In one study, increased contents of $CD68^+$ TAMs were shown to predict longer OS in immunochemotherapy-treated DLBCL patients [221]. In line with these results, higher *CD68* gene expression levels as well as high $CD68^+$ TAM cell contents were reported to associate with longer PFS

and OS among immunochemotherapy-treated DLBCL patients also in another study, and the prognostic impact on OS was independent of aaIPI [220]. By contrast, higher $CD163^+$ M2 TAM count and high $CD163^+/CD68^+$ ratio were reported to be associated with shorter OS and PFS among rituximab-treated DLBCL patients [219,221]. Interestingly, higher content of $CD68^+$ TAMs either had no correlation with prognosis or associated with worse survival among DLBCL patients treated without rituximab, indicating a dual, treatment-specific role of TAMs [220,222]. Additionally, extended extranodal involvement, defined as two or more extranodal sites, has been reported to be more common in patients with high M2 TAM count [219].

Based on current knowledge on tumor biology and tumor immunity, the role of immune checkpoint inhibitors and other TME-targeting therapies has been widely studied in solid tumors and lymphomas including DLBCL [472-475]. Their role in the treatment of T-DLBCL has not been established; however, preliminary data on PD-1 blockade in one T-DLBCL patient with CNS relapse has shown promising results, with further studies ongoing [476].

3 Aims of this study

T-DLBCL is a rare and aggressive lymphoid malignancy with dismal prognosis and a high recurrence rate. Due to the lack of prospective randomized clinical trials the evidence on different treatment modalities has been frail. The role of TME, more specifically TAMs, TILs, and T-cell exhaustion, has been uncharacterized. The aim of this study was to evaluate treatment results of T-DLBCL patients and determine the clinical impact of TME in T-DLBCL.

The specific aims of this work were:

1. to analyze the effect of different treatment modalities on the survival and CNS relapse rate of patients with T-DLBCL in a Nordic patient cohort (I).
2. to study the cellular and molecular immunological profiles of the tumor tissue of T-DLBCL patients and associate the findings with patient survival (II).
3. to characterize different TIL phenotypes and their possible association with clinical risk factors and survival in patients with T-DLBCL (III).
4. to investigate whether the relative expression of immune checkpoint molecules by tumor cells and tumor-infiltrating immune cells associates with survival in patients with T-DLBCL (IV).

4 Material and methods

This thesis was conducted as a Nordic collaboration study between Denmark and Finland with patients from the Danish lymphoma registry and three university hospitals in Southern Finland.

4.1 Patients

This thesis work includes four retrospective population-based substudies (I-IV), one of which was conducted as a Nordic collaborative study (substudy I). T-DLBCL patients diagnosed between the years 1987–2013 were searched from the Danish lymphoma registry and the pathology databases of three university hospitals in southern Finland: Helsinki, Tampere, and Turku University Hospitals (substudy I) [477]. Three translational studies (substudies II-IV) were conducted on tumor tissue of the same T-DLBCL patients identified from the pathology databases of Helsinki, Tampere, and Turku University Hospitals. Only patients with DLBCL and lymphoma involvement of the testis at diagnosis were included. Patients with primary CNS lymphoma involvement at diagnosis were excluded.

4.1.1 Nordic collaborative study (I)

For the patient selection in the Nordic collaborative study, T-DLBCL patients were searched from the Danish lymphoma registry and the pathology databases of three university hospitals in southern Finland (see section 5.1). Clinical data was collected from the Danish national lymphoma registry (Danish patients) and patient records (Finnish patients) [477].

4.1.2 Translational studies (II-IV)

Formalin-fixed, paraffin-embedded (FFPE) tumor tissue biopsies of primary diagnostic orchiectomy samples were collected from local biobanks for the Finnish patients included in this study. The samples were utilized in translational studies (substudies II-IV) with minor variations.

With the aim of exploring cellular and molecular immunological profiles in actively treated T-DLBCL patients, we selected the patients treated with anthracycline-based chemotherapy for the analysis (substudy II). For comparison, we used a dataset of primary DLBCL patients from the database of Genotypes and Phenotypes study accession: phs000532.v2.p1 (CGCI) [478].

To study TAM and TIL contents and to further characterize their immunophenotypes and to associate the findings with disease characteristics and survival, we included all Finnish T-DLBCL patients with tumor tissue available in these studies (substudies III-IV).

4.2 Study design and biological analyses

Data of T-DLBCL patients was retrospectively collected and organized for the analyses. Different treatment modalities as well as baseline clinical factors were recorded. The clinical patient data was utilized in all four substudies of this thesis (I-IV).

For the Finnish patients, diagnostic FFPE tumor tissue biopsies from primary orchiectomy samples were collected from the local biobanks. Lymphoma diagnoses were re-evaluated by experienced hematopathologists to match the latest revised Fourth Edition of the WHO classification of Tumours of Haematopoietic and Lymphoid Tissues [6]. TMA was constructed from FFPE tumor samples, and the cores were selected by the hematopathologist. The samples were used for analysis in all four original publications of this thesis (substudies I-IV).

Gene expression analysis was used for molecular immunological profiling (substudies II and IV). IHC and multiplex immunohistochemistry (mIHC) were conducted to study the cellular immunological profiles (substudies I-IV). mIHC was utilized in studying TIL, TAM, and tumor cell immunophenotypes as well as the PD-1–PD-L1 pathway (substudies II-IV).

Lymphoma stage was defined using the Ann Arbor classification, and COO was determined according to the Hans algorithm (substudies I-IV) [65,96,479].

4.2.1 Immunohistochemistry (I-II)

IHC was conducted on TMA slides according to the manufacturer's instructions. After deparaffinization, heat-induced epitope retrieval (121°C, 3 minutes), and blocking of endogenous peroxidase, TMA sections were incubated with Abs. A list of all generally validated Abs used in substudies I-IV is provided in Table 8. Stainings were completed with Vectastain ABC-HRP (Peroxidase) Kit reagents (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions, and slides were counterstained with hematoxylin.

The samples were scored positive for CD10, Bcl-6, and MUM-1 if 30% or more of the tumor cells were stained with the Ab. For Bcl-2, the cutoff level was 50%. In substudy II, patients were divided into three subgroups based on Anti-HLA class I ABC Ab, Anti-HLA class II DR Ab, and B2M IHC stainings: patients with membranous staining in the majority (>90 %) of the tumor cells were classified as highly positive (normal). Patients with no membranous staining were stratified as negative. The cases with mixed cytoplasmic and membranous staining were classified as moderately positive. Highly positive and moderately positive groups were merged when defining the triple-positive group.

Table 8. Antibodies and mIHC panels.

IHC (substudy, I-IV)					
I	CD79[a]*	CD10*	Bcl-2*	Bcl-6*	MUM-1*
II	HLA-ABC	HLA-DR	B2M		
Theme of mIHC (substudy, I-IV)	**GFP/FITC (green)**	**Cy3 (blue)**	**Cy5 (red)**	**Cy7 (white)**	**Panel number**
T-cells and NK cells (II)	CD56	$CD3_1$	$CD8_1$	$CD4_1$	1
T_{reg}s and Th1 cells (III)	T-bet	$CD3_2$	FoxP3	$CD4_2$	2
Exhausted $CD3^+$ and $CD4^+$ T-cells (III)	$CD4_3$	$CD3_2$	TIM-3	LAG-3_1	3
Activated $CD8^+$ T-cells (III)	GrB	OX40	Ki-67	$CD8_2$	4
Exhausted $CD8^+$ T-cells (III)	LAG-3_2	PD-1^+	TIM-3	$CD8_2$	5
T-cells (IV)	PD-1_2	$CD3_1$	$CD8_1$	$CD4_1$	6
Macrophages (IV)	c-MAF	PD-L1	PD-L2	CD68	7
B-cells and macrophages (IV)	PD-1_2	PD-L1	CD163	CD20	8

[a]Abs: HLA-ABC (clone EMR8-5) Abcam, HLA-DR (clone LN3) Abcam, B2M (clone A0072) Dako, CD56 (clone MRQ-42) Cell Marque Rocklin CA, $CD3_1$ (clone EP449E) Abcam, $CD8_1$ (clone C8/114B) Abcam, $CD4_1$ (clone EPR6855) Abcam, T-bet (clone 91109) Abcam, $CD3_2$ (clone MA5-14482) Thermo, FoxP3 (clone 20034) Abcam, $CD4_2$ (clone ab133616), $CD4_3$ (clone MA5-12229) Thermo, TIM-3 (clone 45208) CST, LAG-3_1 (clone C18692) Lsbio, GrB (clone ab4059) Abcam, OX40 (clone 14-1347-82) Thermo, Ki-67 (clone 9106-S0) Thermo, $CD8_2$ (clone M7103) Dako, LAG-3_2 (clone 180187) Abcam, PD-1_1 (clone B12784) LSBio, PD-1_2 (clone PDCD1) LsBio, c-MAF (clone EPR16484) Abcam, PD-L1 (clone E1L3N) Cell Signaling, PD-L2 (polyclonal) Sigma, CD68 (clone KP1) Abcam, CD163 (clone EPR14643) Abcam, CD20 (clone L26) BioSB; *Validated standard Ab in routine clinical use; GFP, Green fluorescent protein; FITC, Fluorescein isothiocyanate; Cy, Cyanine.

4.2.2 Multiplex immunohistochemistry (II-IV)

mIHC is a technique combining fluorescent and chromogenic stainings with DAPI counterstain to detect the nucleus, enabling the detection of four different markers in one cell simultaneously. The Abs and panels used in mIHC are listed in Table 8.

First, TMA blocks were cut in 3.5 µm sections, dried overnight, and stored for short-term use at +4°C. Protein blocking and Ab incubations were performed in a humid chamber. TMA slides were deparaffinized in xylene and rehydrated in graded ethanol series and H_2O. Heat-induced epitope retrieval (HIER) was carried out in 10 mM Tris-HCl–1 mM EDTA buffer (pH 9) in +99°C for 20 min (PT Module, Thermo Fisher Scientific, Waltham, MA). Peroxide activity was blocked in 0.9% H_2O_2 solution for 15 min. Protein block was performed with 10% normal goat serum (TBS-NGS) for 15 min. Slides were washed three times with 0.1% Tween-20 (Thermo Fisher Scientific) diluted in 10 mM Tris-HCL buffered saline pH 7.4 (TBS) after peroxide block, Ab incubations, and fluorochrome reaction.

Primary Abs were diluted in protein blocking solution and incubated for 1 h 45 min. Secondary anti-mouse or anti-rabbit horseradish peroxidase-conjugated (HRP) Abs (Immunologic, Netherlands) diluted 1:1 with washing buffer were applied for 45 min. Tyramide signal amplification (TSA) Alexa Fluor 488 (PerkinElmer, Waltham, MA) diluted in TBS was applied for 10 min.

Primary Abs were denaturated and enzymatic activity of secondary Ab HRP was quenched by repeating HIER. After this, peroxide and protein block were repeated, followed by application of a different primary Ab, and matching HRP-conjugated secondary Ab diluted 1:3 with washing buffer and TSA Alexa Fluor 555 (PerkinElmer). Thereafter, HIER, peroxide block, and protein block were again repeated. Subsequently, the slides were incubated with two additional primary Abs immunized in different species overnight in +4°C. Next, AlexaFluor647 and AlexaFluor750 fluorochrome-conjugated secondary Abs (Thermo Fisher Scientific) diluted in 1:150 and DAPI (Roche) counterstain diluted 1:250 in washing buffer were applied for 45 min. Finally, ProLong Gold mountant (Thermo Fisher Scientific) and a coverslip were applied on the slides.

With the aim of minimizing false positive signal from Ab cross-reactions during the mIHC procedure, primary Abs selected for mIHC were required to be completely denatured during the HIER step between staining rounds. This was assured by examining the denaturation properties of all primary Abs by performing an additional HIER step between primary and secondary Ab incubation. Abs not denaturing completely were detected with Cy5 and Cy7 fluorescence probes, which do not require denaturation.

Fluorescent images were established with AxioImager.Z2 (Zeiss, Germany) microscope equipped with a Zeiss Plan-Apochromat 20x objective (NA 0.8, papers II and IV) or EC Plan-Neofluar 20x objective (NA 0.8, paper III), CoolCube1 CCD camera (MetaSystems, Germany), PhotoFluor LM-75 (89 North) metal-halide light source, and Zeiss EPLAX VP232-2 power supply. DAPI, FITC, Cy3, Cy5, and Cy7 filters with compatible LED light sources were used and exposure times for all fluorescence channels were optimized visually for fluorescence imaging. Scanned images were acquired and converted to JPEG2000 format (95% quality) for image analysis to enable reduction in memory demand.

In the image analysis, the quality of gray-scale images of each TMA spot was first assessed. Few images were discarded due to blurred focusing or unsuccessful image registration caused mainly by air bubbles in mounting media or shattered tissue, respectively. DAPI-counterstained nuclei were segmented with adaptive Otsu thresholding, clumped objects separated by intensity patterns, and cells segmented with nuclei contour expansion. Machine-learning platform CellProfiler 2.1.2 and 2.2.0 was used for cell segmentation, intensity measurements (upper quartile intensity), and immune cell classification [480]. Single-cell analysis software FlowJo v10 (FlowJo LLC.) was used for computing the marker co-localization. The optimal gate coordinates were ensured by visualizing matching cells with CellProfiler.

Spots with fewer than 5,000 cells were excluded from the analyses, and data from duplicate spots of the same patient were merged by using the mean value. The proportions of different immune cell subtypes were defined as either the proportion of all cells (e.g. $CD3^+CD4^+$ cells implying the proportion of $CD3^+CD4^+$ cells of all the cells in a TMA spot) or the proportion of distinct immune cell subtype (e.g. $PD\text{-}1^+CD3^+/CD3^+$ implying the proportion of $PD\text{-}1^+CD3^+$ cells of all $CD3^+$ cells in a TMA spot).

4.2.3 Gene expression analysis (II, IV)

Gene expression analysis was performed using Nanostring nCounter Human PanCancer Immunoprofiling Panel (XT-CSO-HIP1-12, NanoString Technologies, Seattle, WA). The total

RNAs were isolated with RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Life Technologies, Thermo Fisher Inc., Waltham, MA). Two or three 20 µm sections were cut from the FFPE blocks and processed according to the manufacturer's instructions. A total of 100 ng of RNA was hybridized overnight at 65°C with the Human PanCancer Immunoprofiling Panel codesets (XT-CSOHIP1-12, NanoString Technologies, Seattle, WA). Purification and binding of the hybridized probes to the cartridge were performed on the nCounter Prep Station, followed by scanning the cartridge on the nCounter Digital Analyzer (Nanostring Technologies).

The data were analyzed with nSolver 3.0 software (NanoString Technologies). The quality of the data was confirmed by using the default QC settings, and normalization was done using the geNorm algorithm1. The data were log2-transformed for subsequent analyses. For hierarchical clustering, the data were z-score-transformed, and clustering was done with the JExpress 2012 software2 using Euclidean distance with average linkage. Pathway analysis was done with DAVID Bioinformatics Resources 6.83, using genes from the PanCancer Immunoprofiling Panel as a background.

4.3 Statistical methods

In all the substudies included in this thesis, OS was defined as time between diagnosis and last follow-up or death from any cause, DSS as time between diagnosis and death related to lymphoma, and PFS as time between diagnosis and lymphoma progression or death from any cause.

Differences in the baseline categorical biological and clinical prognostic factors were assessed by using the Chi square test. Mann-Whitney U and Kruskal-Wallis tests were used when comparing the frequencies between two or more groups, respectively. The prognostic values of different factors were studied with Cox univariate regression analysis. Multivariate analyses were performed according to the Cox proportional hazards regression model. Survival rates were estimated using the Kaplan-Meier method (log-rank test). The correlations were tested with Spearman's rank correlation. The potential bias due to duration of follow-up was assessed by Schoenfeld residual. Hierarchical clustering was conducted with JExpress 2012 Software using Euclidean distance with average linkage [481]. Pathway analysis was done with DAVID Bioinformatics Resources 6.8 using genes from the PanCancer Immunoprofiling Panel as a background [482,483].

Statistical analyses of all the substudies in this thesis were performed using IBM SPSS version 24.0 (IBM, Armonk, NY). Probability values below 0.05 were considered statistically significant. All comparisons and all comparative tests were two-tailed.

4.4 Ethical considerations

This study is a retrospective analysis and did not influence the treatment of patients included in this study. The study protocol and sampling were approved by the Institutional Review Boards, Ethics Committees, and Finnish National Supervisory Authority for Welfare and Health (Valvira, license number 9505/06.01.03.01/2013).

5 Results

5.1 Patient characteristics

5.1.1 Nordic collaborative study (I)

We found altogether 235 patients with T-DLBCL and clinical data available. Among these patients, median age at diagnosis was 71 years (range 37–93 years) and all but two patients had undergone orchiectomy. Of the patients, 40% (n=95) had an isolated lymphoma involvement of one or both testes and were considered as stage I disease; 62% of the cases (n=146) had a limited stage disease (stage I-II) and were considered as primary T-DLBCLs [96]. Lymphoma involvement of the contralateral testis was detected among 11% (n=8) of the Finnish patients (n=75) but this information was not recorded for the Danish patients.

For further analyses, we selected the patients who, in addition to undergoing orchiectomy, had been treated with active systemic (immune-)chemotherapy ((R-)CHOP or (R-)CHOP -like regimen) with curative intent. Patients with concurrent CNS lymphoma involvement at diagnosis (n=7) and two patients with no information available on the cause of death were excluded, resulting in a total of 189 patients with median age at diagnosis of 69 years (range 37–88 years) for further analysis. Less than half of these 189 patients (40%, n=76) had been treated with IV CNS-targeted chemotherapy and 66% (n=124) had advanced stage T-DLBCL. Of the 189 patients, 63% (n=120) had been treated with rituximab whereas 37% (n=69) had been treated in the pre-rituximab era. Baseline patient characteristics of the 189 patients, distributed according to IV CNS-targeted therapy, are summarized in Table 1, substudy I, whereas Table S1, substudy I, distributes the patients according to primary T-DLBCL and advanced stage T-DLBCL [96].

Altogether 91 Finnish patients with T-DLBCL were identified. Among these patients, primary diagnostic T-DLBCL tissue was available for further analysis from 79 of the cases. For all 79 cases, the diagnosis of T-DLBCL was confirmed according to the revised Fourth Edition of the WHO classification of Tumours of Haematopoietic and Lymphoid Tissues [6]. Seventy-four of these patients were included in the analysis of biological risk factors in the Nordic collaborative study (substudy I). Of these 74 cases, 81% (n=60) had been treated with a curative intent, and 76% (n=56) represented non-GCB phenotype DLBCL based on the Hans algorithm [65]. Bcl-2 and Bcl-6 IHC results were available for 61 T-DLBCL patients, with 77% (n=47) positive with Bcl-2 and 38% (n=23) positive with Bcl-6. Data on c-Myc IHC was available for 38 T-DLBCL patients with only one case scored as positive.

5.1.2 Translational studies (II-IV)

All 79 T-DLBCL patients with tumor tissue available were included in the TIL immunophenotype analyses (substudy III), whereas for the analysis on TAMs, TILs, and the PD-1–PD-L1 pathway (substudy IV), few images were discarded due to blurred focusing or unsuccessful image registration, as described earlier in section 4.2.2, resulting in a total of 74 patients. Sixty patients had

been treated with anthracycline-based (immune-)chemotherapy with curative intent and were included in the gene expression profiling and T-/NK cell analysis (substudies II, IV).

The majority of the 60 T-DLBCL patients treated with anthracycline-based (immune-) chemotherapy with curative intent were over 60 years of age at diagnosis, had a limited stage disease, low IPI score, and had received rituximab and IV CNS-targeted chemotherapy as part of their lymphoma treatment. The contralateral testis had been treated (either surgically or by irradiation) only in a minority of the patients. The majority of the cases represented non-GCB phenotype DLBCL. The baseline patient characteristics of these 60 patients are summarized in Table 1, substudy II.

For comparison, we used data of 96 patients with primary DLBCL from the CGCI cohort (substudy II) [478]. The majority of the patients with primary DLBCL were over 60 years of age at diagnosis but, unlike the 60 patients with T-DLBCL, only a minority of the patients with primary DLBCL had a limited stage disease and low IPI score (Table 1, substudy II). A vast majority of the primary DLBCL patients had been treated with a rituximab-containing regimen, and slightly less than half of the cases represented non-CGB phenotype DLBCL. Additionally, one third of the patients were women. The baseline patient characteristics of the 96 primary DLBCL patients are summarized in Table 1, substudy II.

The largest T-DLBCL cohort among the translational studies was the cohort of 79 patients in the TIL immunophenotype analysis (substudy III) with median age at diagnosis being 70 years (range 37–92 years). The baseline patient characteristics did not significantly differ according to stage, IPI score, or COO from those described earlier among the 60 patients treated with curative intent (Table 1, substudy II; Table 1, substudy III). The proportion of patients that had been treated with rituximab and/or IV CNS-targeted chemotherapy was, however, slightly smaller in this larger patient cohort (Table 1, substudy III).

The baseline patient characteristics of the 74 patients included in the analyses of TAMs, TILs, and the PD-1–PD-L1 pathway (substudy IV) were comparable with those of all 79 patients with tumor tissue available (substudy III) and are listed in Table 1, substudy IV, distributed according to the content of PD-L1^{+} TAMs.

5.2 Treatment modalities

The use of different treatment modalities was analyzed in the Nordic collaborative study with 189 T-DLBCL patients (substudy I), all of them treated with anthracycline-based (R-)CHOP or (R-)CHOP-like regimen. As described earlier in section 5.1.1, the majority of these patients had received rituximab, whereas only a minority of them were treated with IV CNS-targeted chemotherapy (HD-Mtx or HD-Ara-C). IT CNS-targeted chemotherapy was given to 33% of the patients (n=63). All patients had undergone orchiectomy, and the contralateral testis was treated in 47% of the patients (n=88), with surgery in 13 and with irradiation in 75 patients.

The use of IV CNS-targeted chemotherapy was as common in the rituximab era as before the introduction of rituximab (43% vs. 36%, p=0.443, respectively). Instead, patients receiving IV CNS-targeted chemotherapy were significantly younger than the ones treated with no CNS-targeted chemotherapy or with IT CNS-targeted chemotherapy only (68% vs. 39% of the patients 70 years or younger at diagnosis, p<0.001, respectively, Table 1, substudy I).

Other baseline patient characteristics such as performance status, stage, IPI score, or COO did not significantly differ between patients receiving IV CNS-targeted chemotherapy or not.

Treatment of the contralateral testis and the use of IT only CNS-targeted chemotherapy were more common in the rituximab era (treatment of the contralateral testis or not, 53% vs. 36%, p=0.035; and IT only CNS-targeted chemotherapy or not, 42% vs. 19%, p=0.014, after and before the introduction of rituximab, respectively) and in Denmark than in Finland (treatment of the contralateral testis or not, 53% vs. 33%, p=0.002; and IT only CNS-targeted chemotherapy or not, 37% vs. 7%, p<0.001, in Denmark vs. in Finland, respectively). Analogously, the contralateral testis was more often treated among patients who had received IT CNS-targeted chemotherapy (treatment of the contralateral testis in 65% vs. 37% of the patients receiving IT CNS-targeted therapy or not, p<0.001, respectively). Only 8% of the patients (n=16) were treated with both IT and IV CNS-targeted chemotherapy, more commonly in combination with rituximab (13 vs. 3 patients, treated with or without rituximab, respectively).

5.3 The molecular immunological profiles

The molecular immunological profiles were studied by analyzing the gene expression levels of 730 immune-associated genes and 40 housekeeping genes from FFPE primary diagnostic lymphoma tissue of 60 T-DLBCL patients with Nanostring nCounter Human PanCancer Immunoprofiling Panel. The results were compared with corresponding data of 96 patients with primary DLBCL.

5.3.1 Molecular immunological profiles in T-DLBCL (II)

Based on an unsupervised hierarchical clustering, we identified three gene signatures differentially expressed between T-DLBCL patients, Figure 1, substudy II. The largest cluster consisted of 121 genes forming a gene signature clearly separating the patients into three subgroups with different expression levels (low, intermediate, and high; Figures 2a and S1a, substudy II). The signature was named "T-lymphocyte signature" based on the genes in the signature that were found to be enriched for T-cell and NK cell markers and signaling (e.g. *CD3D/E/G, CD4*, and *CD8A/B*). A list of all 121 genes in the signature is presented in Table S1, substudy II, and a list of the pathways enriched among these genes is shown in Table 2, substudy II. There were no significant differences in the baseline patient characteristics including age at diagnosis, stage, IPI score, COO, and treatments between the patients with higher and low expression of the T-lymphocyte signature genes (Table S5, substudy II).

Two smaller signatures were named "Cytokine signature I" and "Cytokine signature II" based on the 44 genes enriched for cytokines and 25 genes enriched for cytokines and cytokine receptors, respectively. The genes in Cytokine signatures I and II are listed in Tables S2 and S3, substudy II. The absolute expression levels of Cytokine signature II genes were low, but patients could be divided into two subgroups with higher and lower expression levels and there were no significant differences in the baseline patient characteristics between these two subgroups.

We also analyzed the expression levels of macrophage genes *CD68, CD163,* and *MAF;* B-cell gene *MS4A1* (CD20); and immune checkpoint molecules coding genes *CD274* (PD-L1), *PDCD1LG2* (PD-L2), and *PDCD1* (PD-1). We observed a strong correlation between the expression levels of *CD68* with the expression levels of *CD274* (rs=0.654, p<0.001), *PDCD1LG2* (rs=0.636, p<0.001), *CD163* (rs=0.602, p<0.001), and *MAF* (rs=0.425, p=0.001), and slightly less strong correlation with the expression level of *PDCD1* (rs=0.300, p=0.020). There was no correlation between the expression of *CD68* and *MS4A1*.

5.3.2 Molecular immunological profiles in primary DLBCL (II)

RNA-sequencing data from patients with primary DLBCL was used for comparison. Performing a hierarchical clustering of the gene expressions levels of the T-lymphocyte signature genes, we found a subgroup of patients with low gene expression levels (Figures 2e and S1c, substudy II). When comparing the primary DLBCL patients with high and low expression levels of the T-lymphocyte signature genes, there were no significant differences in the baseline patient characteristics. The baseline patient characteristics of the T-lymphocyte signature patient groups in the primary DLBCL cohort are summarized in Table S5, substudy II. The expression levels of Cytokine signature I and II genes did not significantly differ among patients with primary DLBCL.

5.4 The cellular immunological profiles

IHC and mIHC were utilized in examining NK cells and the expression of HLA class I and II molecules (substudy II); TIL immunophenotypes (substudy III); and TAMs, TILs, and the PD-1–PD-L1 pathway (substudy IV) from FFPE tumor tissue samples of patients with T-DLBCL.The expression of HLA molecules I and II as well as B2M, a component of HLA I, was determined in order to study the escape of the tumor cells from immunosurveillance. Markers CD3 and CD8 were used to detect all TILs and CTLs, respectively; CD4 in combination with FoxP3 and T-bet was used to identify T_{reg}s and Th1 cells, respectively. CD68 was used to detect all TAMs, whereas other macrophage markers (CD163 and c-MAF) were used to identify M2-polarized TAMs. CD20 was used to identify lymphoma cells, and CD56 to detect NK cells. Subpopulations of TAMs and TILs were defined by the presence or absence of PD-1, PD-L1, PD-L3, LAG-3, TIM-3, OX40, GrB, and Ki-67. All the panels and Abs are described in more detail in Table 8, section 4.2.1.

5.4.1 TIL immunophenotypes (III, IV)

To get a better overview of the results on specific TIL immunophenotypes, we performed an unsupervised hierarchical clustering of the data studied in substudy III and could see that the expression of all the studied markers combined clearly separated the patients into subgroups with higher and lower expression (Cluster 1, named "T-cell cluster", Figure 1a, substudy III). Analyzing all the exhaustion markers studied in substudy III, the patients could be further divided into subgroups with higher and lower expression of all the exhaustion markers com-

bined (Cluster 2, named "Exhausted phenotype cluster", Figure 1b, substudy III). Additional smaller clusters (named "Cytotoxic activity cluster", "Proliferative cells cluster", and "T_{reg} cluster") could also be observed and are shown in Figure S1, substudy III. There were no significant differences between the high and low subgroups of the T-cell and Exhausted phenotype clusters according to the known clinical and biological risk factors (Table S3, substudy III).

Altogether, great variation in the content of distinct TIL subtypes and their immunophenotypes could be seen between the patients (Figures 2a-d, substudy III). In addition to Ki-67$^+$ CTLs, the most common immunophenotypes were the exhausted TILs. Clearly the most prominent exhaustion marker among both CD4$^+$ TILs and CTLs was PD-1, whereas the content of LAG-3$^+$TIM-3$^+$ double positive TILs was fairly low. The most prominent CD4$^+$ TIL subtype was FoxP3$^+$ T_{reg} while the proportions of T-bet$^+$ Th1 cells were considerably low. A small subpopulation of CD4$^+$ TILs was FoxP3$^+$T-bet$^+$ double positive.

We observed altogether higher proportions of TILs and more specifically, a higher content of activated CTLs among patients with non-GCB phenotype, whereas higher proportions of CD4$^+$ TILs associated with a limited stage disease. The contents of activated and exhausted CTLs were lower among patients with advanced stage disease and B-symptoms. Additionally, some variation in the proportions of distinct TIL phenotypes could be seen according to the patients' age at diagnosis. All significant associations of different TIL subtypes and immunophenotypes with the known clinical and biological risk factors are shown in Figure 3, substudy III.

5.4.2 HLA class I and II expression (II)

We observed that only a minority of the T-DLBCL patients showed highly positive membranous staining with HLA-ABC (HLA class I), HLA-DR (HLA class II), and B2M (28%, 17%, and 13%, respectively).

5.4.3 TAMs, NK cells, and the PD-1–PD-L1 pathway (II, IV)

Correspondingly to TILs, the content of other studied immune cell subtypes varied greatly between the patients (Figure 1, substudy IV). We observed that not only a large proportion of TAMs but also a major subpopulation of the tumor cells expressed PD-L1, and about half of the TILs expressed PD-1 (Figure 1, substudy IV; see also Table 11 in section 5.4.4). Furthermore, the content of PD-1$^+$ TILs correlated significantly with the content of PD-L1$^+$ TAMs (Table S2, substudy IV). The relative proportions of CD3$^-$CD56$^+$ NK cells were very low (median 0.3%, range 0.0%–57.2%) as was the overall expression of PD-L2 (median 0.1%, range 0.0%–6.4%).

Significantly higher content of PD-L1$^+$ TAMs was observed among patients with a limited stage disease whereas no other significant differences in the known clinical and biological risk factors could be found between patients with different proportions of PD-L1$^+$ TAMs (Table 1, substudy IV).

5.4.4 A summary of the cellular immunological profiles (II-IV)

There was a high agreement with the results of the proportions of distinct immune cell subtypes according to different panels, and for a summary of the analyses in this thesis, the data of different panels were merged by using the mean value of the panels (e.g. the mean proportion of $CD3^+$ cells based on panels 1, 2, 3, and 6) (M.P. et al. unpublished results). The correlations between different panels are listed in Table 9 (M.P. et al. unpublished results).

Table 9. Correlation of the TIL subtypes based on different mIHC panels.

mIHC	rs[a]	p-value
$CD3^+$ panel 1 *vs.* panel 2	0.919	<0.001
$CD3^+$ panel 1 *vs.* panel 3	0.932	<0.001
$CD3^+$ panel 1 *vs.* panel 6	0.972	<0.001
$CD3^+$ panel 2 *vs.* panel 3	0.976	<0.001
$CD3^+$ panel 2 *vs.* panel 6	0.877	<0.001
$CD3^+$ panel 3 *vs.* panel 6	0.882	<0.001
$CD3^+CD4^+$ panel 1 *vs.* panel 2	0.885	<0.001
$CD3^+CD4^+$ panel 1 *vs.* panel 3	0.855	<0.001
$CD3^+CD4^+$ panel 1 *vs.* panel 6	0.982	<0.001
$CD3^+CD4^+$ panel 2 *vs.* panel 3	0.914	<0.001
$CD3^+CD4^+$ panel 2 *vs.* panel 6	0.865	<0.001
$CD3^+CD4^+$ panel 3 *vs.* panel 6	0.801	<0.001
$CD8^+$ panel 4 *vs.* panel 5	0.893	<0.001
$CD3^+CD8^+$ panel 1 *vs.* panel 6	0.926	<0.001
$CD8^+$ panel 4 *vs.* $CD3^+8^+$ panel 1	0.861	<0.001
$CD8^+$ panel 5 *vs.* $CD3^+8^+$ panel 1	0.943	<0.001
$CD8^+$ panel 4 *vs.* $CD3^+8^+$ panel 6	0.732	<0.001
$CD8^+$ panel 5 *vs.* $CD3^+8^+$ panel 6	0.828	<0.001
PD-1^+ panel 5 *vs.* panel 8	0.651	<0.001
PD-L1^+ panel 7 *vs.* panel 8	0.946	<0.001
PD-$1^+CD8^+/CD8^+$ panel 5 *vs.* PD-$1^+CD3^+CD8^+/CD8^+$ panel 6	0.554	<0.001
LAG-3^+ panel 3 *vs.* panel 5	0.533	<0.001
TIM-3^+ panel 3 *vs.* panel 5	0.942	<0.001
[a]Spearman rho. M.P. et al. unpublished results.		

Additionally, as a proof of concept, the expressions of distinct immune cell phenotype markers at the protein level were shown to correlate with the corresponding gene expressions and are listed in Table 10 (M.P. et al. unpublished results).

Table 10. Correlation of the gene expression levels with the corresponding expression on protein level.

mIHC vs. gene expression level	**rs[a]**	**p-value**
mean CD3$^+$ (panels 1, 2, 3, and 6) *vs. CD3D*	0.773	<0.001
mean CD3$^+$ (panels 1, 2, 3, and 6) *vs. CD3E*	0.724	<0.001
mean CD3$^+$ (panels 1, 2, 3, and 6) *vs. CD3G*	0.720	<0.001
mean CD4$^+$ (panels 2 and 3) *vs. CD4*	0.497	<0.001
mean CD8$^+$ (panels 4 and 5) *vs. CD8A*	0.705	<0.001
mean CD8$^+$ (panels 4 and 5) *vs. CD8B*	0.674	<0.001
CD20$^+$ *vs. MS4A1*	0.817	<0.001
CD68$^+$ *vs. CD68*	0.643	<0.001
mean PD-1$^+$ (panels 5 and 8) *vs. PDCD1*	0.701	<0.001
mean PD-L1$^+$ (panels 7 and 8) *vs. CD274*	0.747	<0.001
mean LAG-3$^+$ (panels 3 and 5) *vs. LAG3*	0.533	<0.001
mean TIM-3$^+$ (panel 3 and 5) *vs. HAVCR2*	0.762	<0.001
FoxP3$^+$ *vs. FOXP3*	0.656	<0.001
T-bet$^+$ *vs. TBX21*	0.212	0.108
GrB$^+$ *vs. GZMB*	0.659	<0.001
[a]Spearman rho; p-val. M.P. et al. unpublished results.		

A summary of the median proportions of distinct immune cell subtypes in the TME studied in substudies II-IV is shown in Table 11 (M.P. et al. unpublished results).

Table 11. Median proportions of distinct immune cell subtypes in the TME.

mIHC	median, % (range)
$CD3^+$ T-cells and PD-1	
mean $CD3^+$ (panels 1, 2, 3, and 6)	20.7 (0.0-69.5)
mean $PD\text{-}1^+$ (panels 5 and 8)	6.9 (0.0-53.5)
$FoxP3^+CD3^+/CD3^+$	2.7 (0.2-16.4)
$T\text{-}bet^+CD3^+/CD3^+$	0.1 (0.0-2.3)
$LAG\text{-}3^+CD3^+/CD3^+$	0.3 (0.0-7.0)
$TIM\text{-}3^+CD3^+/CD3^+$	5.2 (0.5-36.2)
$FoxP3^+T\text{-}bet^+CD3^+/CD3^+$	0.0 (0.0-2.0)
$LAG\text{-}3^+TIM\text{-}3^+CD3^+/CD3^+$	0.2 (0.0-5.1)
$CD3^+CD4^+$ T-cells	
mean $CD3^+CD4^+$ (panels 1, 2, 3, and 6)	9.5 (0.0-60.0)
$PD\text{-}1^+CD3^+CD4^+$	12.3 (0.3-65.9)
$FoxP3^+CD3^+CD4^+/CD3^+CD4^+$	10.8 (0.0-50.3)
$T\text{-}bet^+CD3^+CD4^+/CD3^+CD4^+$	0.0 (0.0-6.5)
$LAG\text{-}3^+CD3^+CD4^+/CD3^+CD4^+$	0.2 (0.0-8.6)
$TIM\text{-}3^+CD3^+CD4^+/CD3^+CD4^+$	6.5 (0.1-36.2)
$PD\text{-}1^+CD3^+CD4^+/CD3^+CD4^+$	58.5 (3.8-100.0)
$FoxP3^+T\text{-}bet^+CD3^+CD4^+/CD3^+CD4^+$	0.0 (0.0-6.1)
$FoxP3^-T\text{-}bet^+CD3^+CD4^+/CD3^+CD4^+$	2.2 (0.0-84.7)
$LAG\text{-}3^+TIM\text{-}3^+CD3^+CD4^+/CD3^+CD4^+$	0.1 (0.0-5.0)
$CD8^+$ T-cells	
mean $CD3^+CD8^+$ (panels 1 and 6)	17.3 (0.0-66.0)
$PD\text{-}1^+CD3^+CD8^+$	13.9 (0.3-67.2)
$OX40^+CD8^+/CD8^+$	0.1 (0.0-1.5)
$GrB^+CD8^+/CD8^+$	2.5 (0.0-25.3)
$Ki\text{-}67^+CD8^+/CD8^+$	22.2 (4.7-85.6)
$LAG\text{-}3^+CD8^+/CD8^+$	1.1 (0.0-10.1)
$TIM\text{-}3^+CD8^+/CD8^+$	3.6 (0.5-36.2)
$PD\text{-}1^+CD3^+CD8^+/CD3^+CD8^+$	64.4 (4.1-100.0)
$OX40^+GrB^+CD8^+/CD8^+$	0.0 (0.0-0.9)
$GrB^+Ki\text{-}67^+CD8^+/CD8^+$	0.3 (0.0-5.8)
$OX40^+Ki\text{-}67^+CD8^+/CD8^+$	0.0 (0.0-0.44)
$LAG\text{-}3^+TIM\text{-}3^+CD8^+/CD8^+$	0.3 (0.0-6.6)
$LAG\text{-}3^+PD\text{-}1^+CD8^+/CD8^+$	0.1 (0.0-5.2)
$TIM\text{-}3^+PD\text{-}1^+CD8^+/CD8^+$	0.2 (0.0-5.4)
Macrophages and PD-L1	
$CD68^+$	23.4 (3.4-81.0)
mean $PD\text{-}L1^+$ (panels 7 and 8)	22.5 (0.1-99.8)
$PD\text{-}L1^+CD68^+$	9.4 (0.0-74.4)
$PD\text{-}L1^+CD68^+$	10.5 (0.1-51.6)
$PD\text{-}L1^+CD68^+$	5.0 (0.0-90.5)
$PD\text{-}L1^+CD68^+/CD68^+$	41.4 (0.1-99.4)
$c\text{-}MAF^+CD68^+/CD68^+$	5.6 (0.0-35.4)
$PD\text{-}L1^+c\text{-}MAF^+CD68^+/CD68^+$	2.4 (0.0-21.7)
Lymphoma cells	
$CD20^+$	87.2 (1.4-99.8)
$PD\text{-}L1^+CD20^+$	19.7 (0.1-98.3)
$PD\text{-}L1^+CD20^+/CD20^+$	34.2 (0.1-100)
M.P. et al. unpublished results.	

5.5 Survival analyses

The effect of clinical and biological risk factors as well as different treatment modalities on the survival of T-DLBCL patients was analyzed in the Nordic collaborative study (substudy I). The association of molecular immunological profiles as well as cellular immunological profiles with the survival of T-DLBCL patients was analyzed in the translational studies using gene expression profiling, IHC, and mIHC (substudies II-IV).

5.5.1 Nordic collaborative study (I)

Among the 189 Nordic T-DLBCL patients that had been treated with a curative intent, the median follow-up was 80 months (range 7–192 months). The cause of death was reported in 79% (n=81) of the 102 registered deaths, with lymphoma being the most common cause (56%, n=57). Major non-lymphoma-related causes of death were other malignancies (10%, n=10), cardiovascular failure (7%, n=7), and infections (3%, n=3). The five-year OS, DSS, and PFS rates were 60%, 71%, and 52%, respectively. Age of 70 years or above at diagnosis, advanced stage disease, and high IPI score (IPI 3-5), all known risk factors in DLBCL, associated with worse survival (Figures S1d-l and Table 2, substudy I). When all the factors that had a significant impact on the patient outcome were included in a multivariate analysis, all other risk factors included in the IPI score except stage remained as independent risk factors for OS and PFS (Table 3, substudy I).

Only 6% of the patients (n=12) experienced CNS relapse as their first recurrence, lymphoma being the cause of death for all but one of them. The risk of CNS relapse was significantly higher among patients with advanced stage disease, higher IPI score, and higher number of extranodal sites of primary lymphoma involvement (Table 2, substudy I). Information on primary adrenal gland or kidney involvement was available for 87% of the patients (n=164) and did not associate with increased risk of CNS relapse (HR 1.507, 95% CI 0.196–11.610, p=0.694) or add to the predictive impact of the IPI score. Additional 6 cases of CNS progression at later relapses were observed, with lymphoma being the cause of death for all of these patients.

5.5.1.1 Different treatment modalities

In univariate analyses, IV CNS-targeted chemotherapy as well as treatment of the contralateral testis were shown to have significant association with better survival of T-DLBCL patients (Figures 1d-f and 1j-l and Table 2, substudy I). Analyzed together in multivariate analysis with age, stage, LHD, the number of extranodal sites involved at diagnosis, and performance status, both IV CNS-targeted chemotherapy and treatment of the contralateral testis remained as independent prognostic factors for OS, DSS, and PFS (Table 3, substudy I). The difference on survival according to whether the patients had received IV CNS-targeted chemotherapy or not remained significant also when adjusted for age (< or ≥ 70 years at diagnosis) and was especially significant among elderly patients (five-year OS, DSS, and PFS rates 81% vs. 36%, 87% vs. 57%, and 66% vs. 30%, for patients ≥70 years at diagnosis treated with or without IV CNS-targeted chemotherapy, respectively). The addition of rituximab significantly improved

DSS among chemotherapy-treated patients with high IPI score (five-year DSS 44% vs. 14%, immunochemotherapy-treated (n=39) vs. chemotherapy-treated (n=14) patients with IPI score 3–5, respectively, p=0.019). There was no significant association with survival with the use of IT CNS-targeted chemotherapy. The number of CNS relapses as first recurrence of lymphoma was altogether low in our patient material (6%), and no association to survival with different treatment modalities could be shown.

5.5.1.2 COO and other biological risk factors

Non-GCB phenotype had a significant association to inferior survival with 5-year PFS of 53% vs. 87%, p=0.05, for the non-GCB vs. GCB phenotypes, respectively. We could also observe a trend towards worse prognosis among cases with Bcl-2 positivity (5-year OS 60% vs. 83%, p=0.118; 5-year DSS 73% vs. 90%, p=0.105; 5-year PFS 57% vs. 76%, p=0.112, for Bcl-2 positive vs. Bcl-2 negative cases, respectively). Bcl-6 positivity had no association with patient survival, whereas with only one c-Myc positive case, no analyses on its possible impact on survival could be conducted.

5.5.2 Translational studies (II-IV)

The effect of molecular immunological profiles on the survival of T-DLBCL patients was analyzed and compared with the corresponding data of primary DLBCL patients (substudy II). The association of TIL and TAM subtypes and their immunophenotypes with the survival of T-DLBCL patients was also studied (substudies III-IV, and IV, respectively). Additionally, the association of HLA class I and II expression with the survival of T-DLBCL patients was analyzed (substudy II). Among the largest patient population of these translational studies (n=79, substudy III) we observed altogether 38 deaths, 27 lymphoma deaths, and 42 lymphoma progressions during the median follow-up of 46 months (range 0–120 months). Correspondingly, the five-year OS, DSS, and PFS rates were 54%, 60%, and 46%, respectively.

5.5.2.1 T-cell signature and Cytokine signatures (II)

Patients with low (n=12) expression of the T-lymphocyte signature genes had significantly shorter OS, DSS, and PFS than patients with intermediate (n=32) and high (n=16) gene expression levels (log-rank p=0.009, p=0.003, and p=0.041, respectively). The adverse prognostic impact of the low expression of the T-lymphocyte signature genes remained significant in multivariate analyses with IPI and was especially evident among patients treated with rituximab (Figures 2c-d and S2b and Table S4, substudy II). In univariate analyses with continuous variables, 72 of the 121 genes in the T-lymphocyte signature, e.g. T-cell surface markers *CD3D/E/G, CD4*, and *CD8A/B*, had a significant association with survival.

Patients with higher expression of the Cytokine signature II genes had significantly shorter five-year PFS than patients with lower or no expression of the signature genes (36% vs. 66%, p=0.005), and the difference also remained significant in multivariate analysis with IPI (HR=3.393, CI95=1.531–7.521, p=0.003). No association with survival was found with the expression of the Cytokine I signature genes.

Primary DLBCL patients with low expression of the T-lymphocyte signature genes had significantly shorter survival than patients with higher expression (PFS p=0.007, OS p=0.034, Figure 2f, substudy II), and the association with PFS remained significant in multivariate analysis with IPI (HR=2.560, CI95=1.151–5.695, p=0.021). No association with survival was observed with the expression levels of Cytokine signature I and II genes among patients with primary DLBCL.

5.5.2.2 TIL content and immunophenotypes, HLA class I and II expression (II-IV)

Higher content of $CD3^+$ TILs in general, $CD4^+$ TILs, and CTLs translated into better survival of T-DLBCL patients when analyzed in univariate analysis with continuous variables (Figure S5 and Table 3, substudy II; see also Figures 10 and 11 in section 5.5.2.4). The favorable prognostic impact of high TIL content remained significant in multivariate analyses with IPI, and consistent with the findings on the gene expression level, the association of high proportions of $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes with longer survival was especially evident among patients treated with rituximab (Figure S6 and Table 3, substudy II; see also Table 12 in section 5.5.2.4).

Among patients with T-DLBCL, the expression of *HLA* class I and II genes was significantly lower within patients in the low T-lymphocyte signature subgroup and worse prognosis ($p<0.05$ for both, Figure S7a, substudy II), and the same was seen among patients with primary DLBCL (Figure S7b, substudy II). No association with survival was seen with the content of $CD56^+$ cells.

Analyzing all the studied TIL markers combined in substudy III, we observed that higher expression of the T-cell cluster markers translated into better survival of T-DLBCL patients (group A, Cluster 1, Figures 1a and 4a-c, substudy III). The favorable prognostic impact remained significant also in multivariate analysis with IPI (see Table 12 in section 5.5.2.4). Unexpectedly, patients with higher expression of the Exhausted phenotype cluster markers also tended to have longer survival (group C, Cluster 2, Figures 1b and 4d-f, substudy III), although the difference did not remain as an independent prognostic factor in multivariate analysis with IPI (see Table 12 in section 5.5.2.4).

The possible association of distinct TIL subtypes and their immunophenotypes with the survival of T-DLBCL patients was analyzed using univariate analysis with continuous variables (Figure 5, substudy III; see also Figures 10 and 11 in section 5.5.2.4). Patients with overall higher content of $PD\text{-}1^+CD3^+CD4^+$ TILs and $PD\text{-}1^+CD3^+CD8^+$ CTLs had significantly longer survival, whereas other distinct TIL immunophenotypes could not be associated with better prognosis (Figure 2b-c, substudy IV; see also Figures 10 and 11 in section 5.5.2.4). The prognostic value of $PD\text{-}1^+$ TILs remained significant in multivariate analysis with IPI and among patients treated with rituximab (Figures S2b-c and Table 4, substudy IV; see also Table 12 in section 5.5.2.4). Despite the overall favorable prognostic impact of high TIL content and the generally low proportions of $T\text{-}bet^+$ TILs, higher proportions of $T\text{-}bet^+$ TILs correlated significantly with adverse patient outcome (Figures 5 and 6a-c, substudy III). Furthermore, the content of $FoxP3^+T\text{-}bet^+$ double positive TILs was shown to be a significant adverse prognostic factor, remaining independent in multivariate analysis with IPI, and significant among patients treated

with rituximab (OS, HR=1.778, CI95=1.128–2.803, p=0.013) (Figures 5 and 6d-f and Table 2, substudy III; see also Table 12 in section 5.5.2.4). Interestingly, the proportion of FoxP3$^-$T-bet$^+$ TILs did not have an association with survival (see Figures 10 and 11 in section 5.5.2.4).

5.5.2.3 PD-L1$^+$ TAM content (IV)

In univariate analyses with continuous variables, higher content of PD-L1$^+$ cells, higher proportion of PD-L1$^+$CD68$^+$ TAMs from all cells, and higher proportion of PD-L1$^+$CD68$^+$ TAMs from all TAMs (PD-L1$^+$CD68$^+$/CD68$^+$) had a significant association with longer survival of T-DLBCL patients (Figure 2a and Table 3, substudy IV). The association of PD-L1$^+$CD68$^+$ content with survival remained as an independent prognostic factor in multivariate analysis with IPI and was also evident among patients treated with rituximab (Figure S2a and Table 4, substudy IV). No association with survival was seen with the content of PD-L1$^+$CD68$^-$ cells or PD-L1$^+$c-MAF$^+$CD68$^+$ TAMs.

5.5.2.4 A summary of the survival analysis of the translational studies (II-IV)

A summary of the association of different clusters as well as distinct TIL subtypes, their immunophenotypes, and PD-1$^+$ cells with the OS and DSS of T-DLBCL patients is shown in Figures 8-9 and Figures 10-11, respectively (M.P. et al. unpublished results). A summary of the association of TAMs, lymphoma cells, and PD-L1$^+$ cells with the OS and DSS of T-DLBCL patients is shown in Figures 12-13 (M.P. et al. unpublished results). A summary of the multivariate analysis of the different signatures and the distinct immune cell subtypes with IPI is shown in Table 12 (M.P. et al. unpublished results).

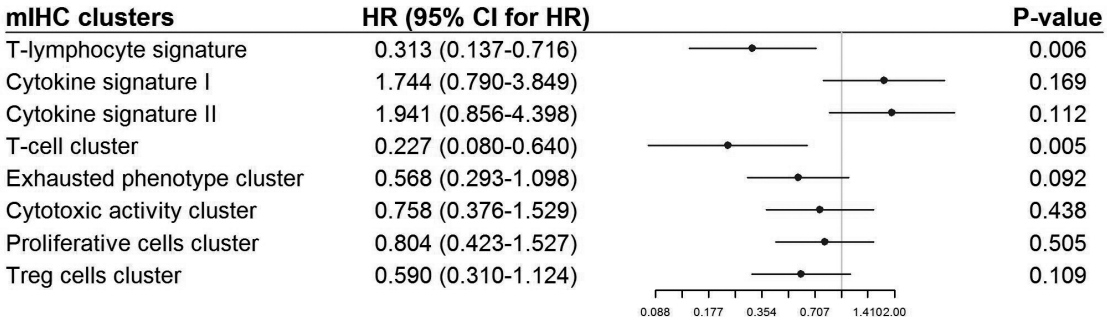

Figure 8. The association of different clusters with OS of T-DLBCL patients. A forest plot with univariate analysis showing the association of higher expression of T-lymphocyte signature genes and T-cell cluster markers with significantly longer OS of T-DBCL patients.

M.P. et al. unpublished results.

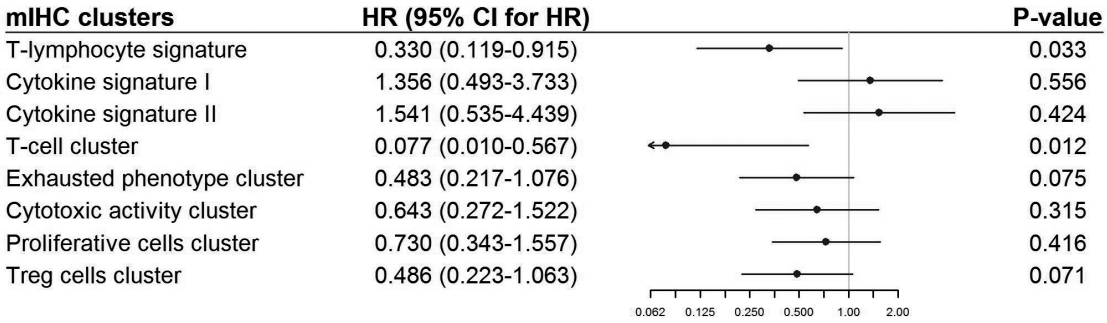

Figure 9. The association of different clusters with DSS of T-DLBCL patients. A forest plot with univariate analysis showing the association of higher expression of T-lymphocyte signature genes and T-cell cluster markers with significantly longer DSS of T-DBCL patients.

M.P. et al. unpublished results.

CD3+ T-cells and PD-1	HR (95% CI for HR)	P-value
mean CD3+ (panels 1, 2, 3 and 6)	0.970 (0.949-0.991)	0.006
mean PD-1+ (panels 5 and 8)	0.989 (0.963-1.016)	0.414
FoxP3+CD3+/CD3+	0.974 (0.878-1.082)	0.627
T-bet+CD3+/CD3+	3.345 (1.422-7.870)	0.006
LAG-3+CD3+/CD3+	1.167 (0.917-1.485)	0.209
TIM-3+CD3+/CD3+	1.004 (0.955-1.055)	0.882
FoxP3+T-bet+CD3+/CD3+	3.966 (1.495-10.522)	0.006
LAG-3+TIM-3+CD3+/CD3+	1.355 (0.991-1.852)	0.057
CD3+CD4+ T-cells		
mean CD3+CD4+ (panels 1, 2, 3 and 6)	0.958 (0.925-0.992)	0.016
PD-1+CD3+CD4+	0.976 (0.953-1.000)	0.050
FoxP3+CD3+CD4+/CD3+CD4+	0.983 (0.947-1.019)	0.345
T-bet+CD3+CD4+/CD3+CD4+	1.529 (1.130-2.069)	0.006
LAG-3+CD3+CD4+/CD3+CD4+	1.107 (0.886-1.384)	0.371
TIM-3+CD3+CD4+/CD3+CD4+	1.010 (0.971-1.051)	0.603
PD-1+CD3+CD4+/CD3+CD4+	0.992 (0.979-1.006)	0.266
FoxP3+T-bet+CD3+CD4+/CD3+CD4+	1.545 (1.114-2.145)	0.009
FoxP3-T-bet+CD3+CD4+/CD3+CD4+	4.833 (0.623-37.456)	0.132
LAG-3+TIM-3+CD3+CD4+/CD3+CD4+	1.319 (0.958-1.815)	0.089
CD8+ T-cells		
mean CD3+CD8+ (panels 1 and 6)	0.965 (0.943-0.988)	0.003
PD-1+CD3+CD8+	0.969 (0.944-0.994)	0.015
OX40+CD8+/CD8+	0.586 (0.177-1.943)	0.382
GrB+CD8+/CD8+	1.014 (0.961-1.069)	0.616
Ki-67+CD8+/CD8+	1.005 (0.990-1.020)	0.525
LAG-3+CD8+/CD8+	0.852 (0.686-1.058)	0.146
TIM-3+CD8+/CD8+	1.008 (0.950-1.071)	0.784
PD-1+CD3+CD8+/CD3+CD8+	0.990 (0.977-1.004)	0.160
OX40+GrB+CD8+/CD8+	0.021 (0.000-12.366)	0.235
GrB+Ki-67+CD8+/CD8+	1.139 (0.834-1.555)	0.413
OX40+Ki-67+CD8+/CD8+	0.031 (0.000-11.535)	0.250
LAG-3+TIM-3+CD8+/CD8+	0.704 (0.428-1.159)	0.167
LAG-3+PD-1+CD8+/CD8+	0.813 (0.447-1.481)	0.499
TIM-3+PD-1+CD8+/CD8+	0.876 (0.542-1.418)	0.591

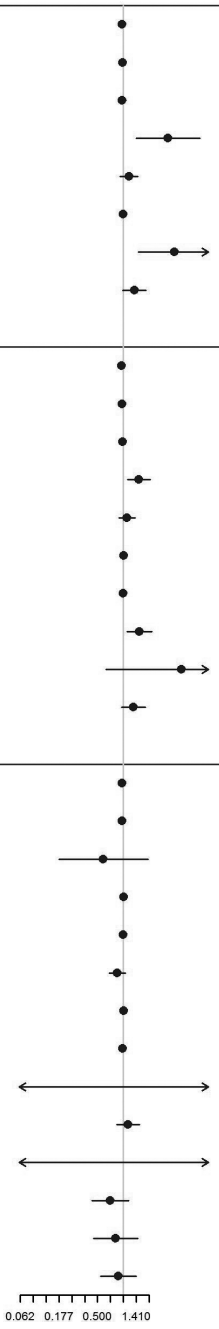

Figure 10. The association of distinct TIL subtypes, their immunophenotypes, and PD-1$^+$ cells with OS of T-DLBCL patients. A forest plot with univariate analysis using continuous variables showing the association of higher proportion of CD3$^+$ TILs in general, CD4$^+$ TILs, CD8$^+$ CTLs, PD-1$^+$ CD4$^+$ TILs as well as PD-1$^+$ CTLs with longer OS of T-DLBCL patients. Higher proportion of T-bet$^+$ and FoxP3$^+$T-bet$^+$ TILs from all CD3$^+$ TILs as well as from all CD4$^+$ TILs translated into significantly shorter OS of T-DLBCL patients. The proportion of FoxP3$^-$T-bet$^+$ TILs from all CD4$^+$ TILs did not have significant association with survival. M.P. et al. unpublished results.

CD3+ T-cells and PD-1	HR (95% CI for HR)	P-value
mean CD3+ (panels 1, 2, 3 and 6)	0.960 (0.933-0.988)	0.005
mean PD-1+ (panels 5 and 8)	0.982 (0.951-1.015)	0.285
FoxP3+CD3+/CD3+	0.966 (0.849-1.098)	0.591
T-bet+CD3+/CD3+	3.785 (1.588-9.021)	0.003
LAG-3+CD3+/CD3+	1.188 (0.925-1.526)	0.177
TIM-3+CD3+/CD3+	0.983 (0.922-1.048)	0.596
FoxP3+T-bet+CD3+/CD3+	4.449 (1.667-11.875)	0.003
LAG-3+TIM-3+CD3+/CD3+	1.338 (0.963-1.859)	0.083
CD3+CD4+ T-cells		
mean CD3+CD4+ (panels 1, 2, 3 and 6)	0.935 (0.890-0.982)	0.008
PD-1+CD3+CD4+	0.961 (0.929-0.995)	0.024
FoxP3+CD3+CD4+/CD3+CD4+	0.991 (0.951-1.033)	0.671
T-bet+CD3+CD4+/CD3+CD4+	1.569 (1.151-2.138)	0.004
LAG-3+CD3+CD4+/CD3+CD4+	1.146 (0.914-1.437)	0.237
TIM-3+CD3+CD4+/CD3+CD4+	0.994 (0.946-1.044)	0.805
PD-1+CD3+CD4+/CD3+CD4+	0.989 (0.974-1.004)	0.161
FoxP3+T-bet+CD3+CD4+/CD3+CD4+	1.585 (1.133-2.217)	0.007
FoxP3-T-bet+CD3+CD4+/CD3+CD4+	6.082 (0.765-48.380)	0.088
LAG-3+TIM-3+CD3+CD4+/CD3+CD4+	1.337 (0.965-1.852)	0.081
CD8+ T-cells		
mean CD3+CD8+ (panels 1 and 6)	0.954 (0.925-0.985)	0.003
PD-1+CD3+CD8+	0.955 (0.922-0.989)	0.011
OX40+CD8+/CD8+	0.514 (0.109-2.418)	0.399
GrB+CD8+/CD8+	0.992 (0.922-1.067)	0.822
Ki-67+CD8+/CD8+	1.009 (0.991-1.026)	0.332
LAG-3+CD8+/CD8+	0.829 (0.632-1.087)	0.175
TIM-3+CD8+/CD8+	0.971 (0.887-1.064)	0.532
PD-1+CD3+CD8+/CD3+CD8+	0.987 (0.972-1.002)	0.084
OX40+GrB+CD8+/CD8+	0.024 (0.000-61.482)	0.350
GrB+Ki-67+CD8+/CD8+	0.927 (0.571-1.504)	0.758
OX40+Ki-67+CD8+/CD8+	0.101 (0.000-48.718)	0.467
LAG-3+TIM-3+CD8+/CD8+	0.600 (0.348-1.254)	0.205
LAG-3+PD-1+CD8+/CD8+	0.803 (0.376-1.715)	0.571
TIM-3+PD-1+CD8+/CD8+	0.910 (0.525-1.577)	0.736

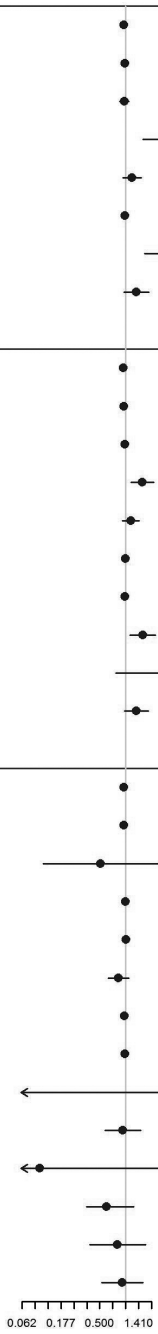

Figure 11. The association of distinct TIL subtypes, their immunophenotypes, and PD-1$^+$ cells with DSS of T-DLBCL patients. A forest plot with univariate analysis using continuous variables showing the association of higher proportion of CD3$^+$ TILs in general, CD4$^+$ TILs, CD8$^+$ CTLs, PD-1$^+$ CD4$^+$ TILs as well as PD-1$^+$ CTLs with longer DSS of T-DLBCL patients. Higher proportion of T-bet$^+$ and FoxP3$^+$T-bet$^+$ TILs from all CD3$^+$ TILs as well as from all CD4$^+$ TILs translated into significantly shorter DSS of T-DLBCL patients. The proportion of FoxP3$^+$T-bet$^+$ TILs from all CD4$^+$ TILs did not have significant association with survival. M.P. et al. unpublished results.

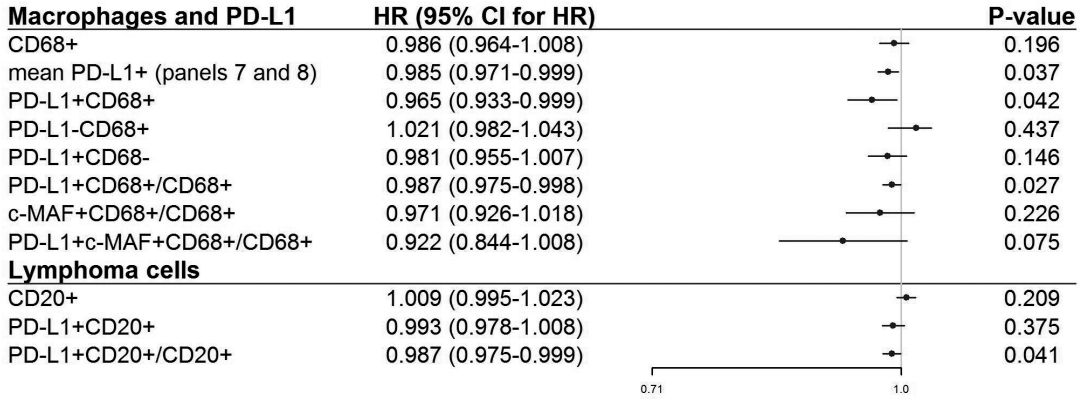

Figure 12. The association of TAMS, lymphoma cells, and PD-L1⁺ cells with OS of T-DLBCL patients. A forest plot with univariate analysis using continuous variables showing the association of PD-L1⁺ cells in general as well as PD-L1⁺ TAMs with longer OS of T-DLBCL patients. No association with survival could be seen with the proportion of PD-L1⁺CD68⁻ cells. However, higher proportion of PD-L1⁺ lymphoma cells from all lymphoma cells as well as higher proportion of PD-L1⁺ TAMs from all TAMs translated into longer OS of T-DLBCL patients.

M.P. et al. unpublished results.

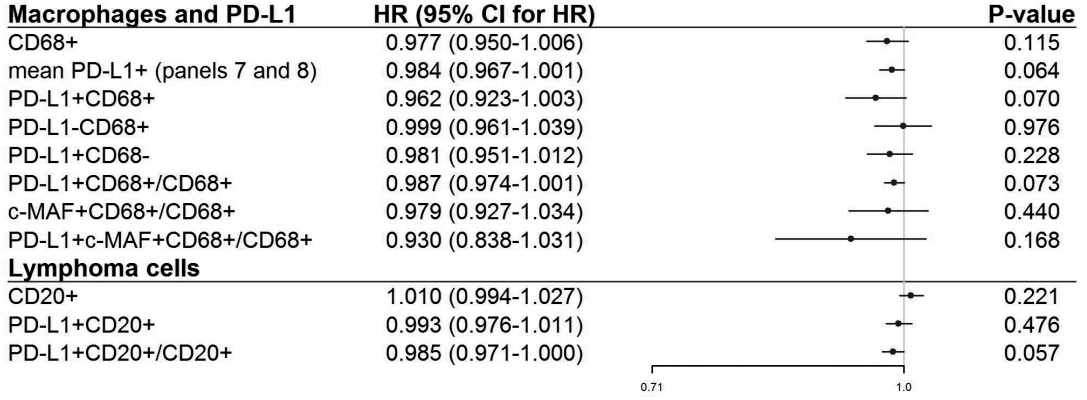

Figure 13. The association of TAMS, lymphoma cells, and PD-L1⁺ cells with DSS of T-DLBCL patients.

A forest plot with univariate analysis using continuous variables showing no significant associations of TAMs, lymphoma cells, and PD-L1⁺ cells with the DSS of T-DLBCL patients.

M.P. et al. unpublished results.

Table 12. Multivariate analysis of distinct signatures and immune cell subtypes with IPI.

Variables	HR[a]	95% CI	p-value
T-lymphocyte signature (low vs. other)	3.267	1.406-7.590	0.006
IPI (3-5)	4.436	1.945-10.117	<0.001
T-cell cluster (group A vs. group B)	0.193	0.066-0.563	0.003
IPI (3-5)	6.382	3.001-13.573	<0.001
Exhausted phenotype cluster (group C vs. group D)	0.851	0.462-1.701	0.649
IPI (3-5)	4.878	2.408-9.787	<0.001
mean $CD3^+$ (panels 1, 2, 3, and 6)	0.967	0.943-0.991	0.007
IPI (3-5)	5.340	2.624-10.866	<0.001
mean $CD3^+CD4^+$ (panels 1, 2, 3, and 6)	0.956	0.919-0.994	0.022
IPI (3-5)	5.272	2.588-10.741	<0.001
mean $CD3^+CD8^+$ (panels 1 and 6)	0.969	0.947-0.992	0.009
IPI (3-5)	5.252	2.583-10.680	<0.001
$PD\text{-}1^+CD3^+CD4^+$	2.654	1.261-5.586	0.010
IPI (3-5)	4.907	2.275-10.585	<0.001
$PD\text{-}1^+CD3^+CD8^+$	2.259	1.075-4.748	0.031
IPI (3-5)	4.971	2.314-10.678	<0.001
$T\text{-}bet^+CD3^+CD4^+/CD3^+CD4^+$	1.525	1.100-2.114	0.001
IPI (3-5)	4.672	2.299-9.494	<0.001
$FoxP3^+T\text{-}bet^+CD3^+CD4^+/CD3^+CD4^+$	4.540	2.238-9.211	<0.001
IPI (3-5)	1.533	1.077-2.181	0.018
$PD\text{-}L1^+CD68^+$	2.214	1.054-4.650	0.036
IPI (3-5)	4.325	2.008-9.312	<0w.001
$PD\text{-}L1^+CD68^+/CD68^+$	2.275	1.054-4.909	0.036
IPI (3-5)	3.608	1.643-7.923	0.001

HR[a], Hazard Ratio; CI, Confidence Interval; IPI, International Prognostic Index. M.P. et al. unpublished results.

6 Discussion

PTL is a rare and aggressive lymphoma entity with a high tendency of CNS relapse and dismal outcome, typically presenting among elderly men and representing DLBCL histologically. Due to the rareness of the disease, no randomized clinical trials have been conducted in T-DLBCL, and the current standard of care is based on retrospective data and few prospective, phase II trials. During the last few years, chromosomal, gene expression profiling, and immunohistochemical studies have led to advancements in the understanding of lymphoma biology and the unique characteristics of T-DLBCL. The role of TME has been the focus of many recent lymphoma studies, and the impact of distinct immune cell subtypes and their immunophenotypes in the TME is gradually beginning to unfold. The characteristics and the impact of TME and the different tumor-infiltrating immune cell phenotypes in T-DLBCL have not, however, been thoroughly studied before.

In the lack of phase III trials, the data presented in this relatively large Nordic collaborative study in substudy I brings important information on the outcome of T-DLBCL patients treated with different treatment regimen and modalities. The findings of translational studies (substudies II-IV) identify a novel T-lymphocyte signature which clearly separates patients with different outcomes and responses to immunochemotherapy as well as present novel information on the content, distribution, and role of TILs, TAMs, and their immunophenotypes in T-DLBCL, further discovering subpopulations of distinct immune cell subtypes that have association with the survival of T-DLBCL patients.

6.1 Clinical data and different treatment modalities

T-DLBCL is known to present with a relatively high incidence of CNS relapses, resulting in poor patient survival [17,85]. Previous literature and clinical practice guidelines therefore recommend the use of CNS prophylaxis as part of the treatment in T-DLBCL [15,85,97,484]. In the lack of phase III trials, the optimal administration of CNS prophylaxis has not, however, been univocally defined, and especially the role of IT CNS-targeted chemotherapy has been controversial. Some retrospective series from the pre-rituximab era have reported improved PFS rates among patients treated with IT CNS-targeted chemotherapy but have not been able to further specify the beneficial role of IT administered chemotherapy [15,85]. Due to lack of evidence, at least in the Nordic countries, the use of IT CNS-targeted chemotherapy is not very common, and the data provided by our study gives more backbone to the decision of not including it in the treatment regimen of T-DLBCL patients [485].

The benefit of IV CNS-targeted chemotherapy in T-DLBCL has not been thoroughly studied. Although one of the conducted phase II trials included IT CNS-targeted chemotherapy as part of the treatment regimen and the other phase II trial included IV CNS-targeted chemotherapy, the results of these two independent studies cannot be compared and no assumptions can be drawn on the benefit of either treatment approach [15,16]. Considering the overall lack of evidence on the optimal administration of CNS-targeted chemotherapy in T-DLBCL, the results of our retrospective analysis showing significantly improved patient survival with

the addition of IV CNS-targeted chemotherapy seem particularly important. Additionally, our findings highlight the essential role of IV CNS-targeted chemotherapy in gaining better systemic control of T-DLBCL, in addition to the aim of reducing the risk of CNS relapse. Notable is also the finding that despite the commonly feared toxicity of IV CNS-targeted chemotherapy in elderly patients, this treatment modality translated into longer survival particularly among patients with advanced age at diagnosis.

The effect of rituximab on the survival of T-DLBCL patients and the risk of CNS relapse in T-DLBCL has not been proven and seems to be controversial [16,67,102,486-489]. However, rituximab is commonly included in the treatment regimen of T-DLBCL and the data from our study supports this policy, especially among high-risk patients. Treatment of the contralateral testis is already commonly considered crucial in the treatment of T-DLBCL patients and the findings in our study highly support this approach [15,101,490]. As a conclusion and based on our findings, the currently recognized standard of care with orchiectomy followed by anthracycline-based immunochemotherapy, IV CNS-targeted chemotherapy, and prophylactic treatment of the contralateral testis can be considered, at the moment, the most recommended first line treatment of T-DLBCL.

6.2 Biological findings and the TME

Previous genetic studies of T-DLBCL have been more or less focused on the COO and different pathway activation -related factors [74,103,127,199,201]. In DLBCL, different genetic signatures such as immune and host response signatures, stromal signatures, and lymphoma driver gene signatures have been shown to separate patients into distinct subgroups with different genetic aberrations, phenotypes, and response to treatment [108,146,193,194,491]. Our aim was to characterize immune-related factors and immune cell phenotypes in T-DLBCL, and we found a novel T-lymphocyte signature with 121 genes enriched for T-lymphocyte markers that could clearly separate T-DLBCL patients with different survival and distinct response to immunochemotherapy. This T-lymphocyte signature was shown to have prognostic impact also in an independent cohort of primary DLBCL patients, further highlighting the importance of the signature genes.

We show that high expression of individual T-lymphocyte surface markers such as *CD3D/E/G*, *CD4*, and *CD8A/B* translated into favorable outcome in T-DLBCL, emphasizing the prognostic role of TILs in the TME of T-DLBCL. As a proof of concept, we observed that the expression of T-lymphocyte surface marker genes correlated well with the expression of the corresponding genes on the protein level, and a higher proportion of TILs associated with significantly improved outcome of patients with T-DLBCL also based on the findings from our mIHC analysis. The favorable prognostic impact was seen with higher content of both $CD4^+$ TILs and CTLs, was independent of IPI, and was especially evident among immunochemotherapy-treate patients. These findings are in line with the previously reported association of high TIL content with better survival of patients with DLBCL, but, to our knowledge, the results on the prognostic impact of TILs in T-DLBCL have not been demonstrated before [213,216,217].

In line with previous findings on the common loss of *HLA* genes in T-DLBCL, we observed only a minority of the T-DLBCL patients' tumor tissue samples showing highly positive membranous staining of HLA class I and II proteins [91,92,204,205]. The previously reported studies

have, however, been conducted with fairly small numbers of T-DLBCL patients and have not been able to report an association of these findings with patient survival. With a considerably larger patient population, we were able to demonstrate that the low expression of *HLA* class I and II genes translated into worse prognosis and lower content of TILs among patients with T-DLBCL. These results further highlight the significant role of TILs in the TME of T-DLBCL and present a possible mechanism of immune evasion caused by disturbed antigen presentation that might lead to impaired recruitment of TILs in T-DLBCL.

In order to further characterize the subtypes of host immune cells in the TME of T-DLBCL, we performed several mIHC panels with different markers to detect distinct TIL and TAM immunophenotypes and the expression of activation and exhaustion markers as well as checkpoint molecules. Altogether, we observed great variation in the distribution of all studied host immune cells among the tumor tissue samples from different patients. Remarkable variation was also seen in the content of lymphoma cell immunophenotypes. To our knowledge, the findings on the host immune and tumor cell immunophenotypes and the variation of their content in T-DLBCL TME have not been previously reported, and our findings bring novel information on the diversity of the disease. We show that higher proportions of checkpoint receptor expressing PD-1$^+$ TILs associate with longer survival of patients with T-DLBCL. On the individual marker level, PD-1 was the only exhaustion marker significantly translating into better patient outcome, and the association with survival was most significant with the proportion to all cells (e.g. PD-1$^+$CD3$^+$CD8$^+$) and less evident when analyzing the proportions of distinct TIL subtypes (e.g. PD-1$^+$CD3$^+$CD8$^+$/CD3$^+$CD8$^+$). In DLBCL, PD-1 expression has been associated with better clinical outcome, but as the studies have not been able to detect the expression of PD-1 to a distinct cell subpopulation, and as PD-1 is known to be expressed on other immune cells as well, the results cannot be directly compared to ours [321,331,467].

Although none of the other studied T-cell exhaustion markers had an association with survival of T-DLBCL patients, when analyzing the studied exhaustion markers combined, a clear trend towards longer survival could be seen among patients with higher overall expression of the exhaustion markers. We assume that this finding, first of all, emphasizes the overall strong favorable prognostic impact of high TIL content more than barely describes an exhausted or de-functioning state of TILs. On the other hand, it seems that the expression of markers that have been considered to describe a state of exhaustion can, perhaps, also demonstrate a certain state of activity of the cell. In line with this hypothesis are the recently published data on PD-1$^+$, LAG-3$^+$, and TIM-3$^+$ triple-positive CD8$^+$ TILs presenting with a highly activated and functional phenotype and negative correlation to tumor burden in murine tumor models [492]. The number and activation of these triple-positive CD8$^+$ TILs were shown to increase in response to anti-PD-L1 therapy [492]. In our patient material, the co-expression of LAG-3 and TIM-3 on CTLs did not have an effect on the outcome of T-DLBCL patients, but when co-expressed on CD4$^+$ TILs, they were shown to have an adverse impact on survival. Previous data on the co-expression of LAG-3 and TIM-3 on CD4$^+$ T-cells are scarce and have been reported in infections such as HIV and some solid tumors [493-496]. Recently published results on colorectal cancer suggest the co-expression identifying a subgroup of potent CD4$^+$ T_{reg}s that suppress the activity of macrophages in the TME [495]. Based on these results, it can be speculated whether the adverse prognostic impact of LAG-3$^+$TIM-3$^+$CD4$^+$ TILs in T-DLBCL could be a result from T_{reg}-mediate downregulation of TAM activity.

Despite the overall favorable prognostic impact of a high TIL content, a subgroup of T-bet$^+$ TILs associated with significantly worse survival of T-DLBCL patients, independently of IPI and especially among immunochemotherapy-treated patients. The overall proportions of T-bet$^+$ TILs were low, and it seems that the presence of even a small number of them is enough to have an adverse impact on patient outcome. Interestingly, the prognostic impact was particularly related to the presence of FoxP3$^+$T-bet$^+$ double positive TILs, and the proportion of FoxP3$^-$T-bet$^+$ TILs did not correlate with patient survival. Based on previous literature, FoxP3$^+$T-bet$^+$ double-positive TILs have been described as T_{reg}s that are optimized for the suppression of Th1 cells [243,252,256]. We assume that the adverse impact of T-bet$^+$ TILs on the outcome of T-DLBCL patients is probably mainly due to the presence of these FoxP3$^+$T-bet$^+$ double-positive Th1 cell suppressive T_{reg}s. The presence of FoxP3$^+$T-bet$^+$ T_{reg}s has also been recognized and associated to tumor growth in lung carcinoma but, to our knowledge, their proper functional role in tumors remains to be solved [497]. Taken together, our findings indicate that despite the overall favorable prognostic impact of TILs, the TME is very complex. The results suggest that distinct subpopulations of TILs, and perhaps more specifically, subpopulations of tumor-infiltrating T_{reg}s, might interact with other host immune cells, leading to suppression of anti-tumor host immunity.

Studying the expression of checkpoint molecules on other tumor-infiltrating immune cells and tumor cells, we observed that altogether higher expression of PD-L1 translated into significantly longer survival of T-DLBCL patients. This association seems to be especially related to the expression on CD68$^+$ TAMs, although also higher proportions of PD-L1$^+$ lymphoma cells seem to be advantageous. Interestingly, the content of PD-L1 expressing CD68$^+$ TAMs correlated with the content of PD-1 expressing TILs. Taking into account previous reports on PD-L1 expression on tumor cells associating with immune escape and T-cell non-responsiveness, as well as promising preliminary results with PD-1 blockade therapy in T-DLBCL, our results seem somewhat paradoxical [209,288,476]. The PD-1–PD-L1 pathways have, however, been shown to be much more complicated, and besides PD-1, also additional binding sites to PD-L1 and PD-L2 have been reported [498-500]. Furthermore, PD-1 expression by TAMs has been shown to inhibit phagocytosis and tumor immunity, and blockade of the PD-1–PD-L1 pathway seems to reverse these impacts [443]. Altogether, our results highlight the diverse and possibly clinically relevant PD-1–PD-L1 signaling and interactions between TAMs and TILs. Since the significant association with survival was seen only with higher proportions of PD-L1$^+$ CD68$^+$ TAMs and not with the content of any other, M2-like, TAM phenotypes, it can be speculated whether the favorable prognostic impact of PD-L1$^+$ TAMs in T-DLBCL might be due to the presence of a subpopulation of M1-like PD-L1$^+$ TAMs. At least it can be assumed that the association with survival is not restricted to M2-like phenotype PD-L1$^+$ TAMs alone. These findings are in line with the previously reported association of CD68$^+$ TAMs with longer survival of patients with DLBCL and bring novel information on the role of TAMs in T-DLBCL since, to our knowledge, no previous reports on the possible prognostic influence of TAMs have been published in T-DLBCL [220,221].

In our patient material, the overall beneficial role of TILs and PD-L1$^+$ TAMs was especially evident among immunochemotherapy-treated T-DLBCL patients and can therefore relate to the crucial role of host immunity and the pre-existing TIL and TAM populations that can induce response to both chemotherapy agents and rituximab. The hypothesis is supported by previ-

ous findings on cyclophosphamide- and doxorubicin-induced anti-tumor immune response, macrophage-involved Ab-dependent cell-mediated cytotoxicity, as well as $CD4^+$ T-cell- and CTL-dependent "vaccinal effect" of rituximab and alterations in the peripheral blood T-cell status upon immunochemotherapy [447-450,453,454,501,502]. Additionally, as HLA proteins are needed in proper antigen presentation and tumor cell recognition, loss of HLA protein expression may, in addition to facilitating lymphoma cells to escape from immunosurveillance, also lead to impaired recruitment of TILs and inadequate response to therapeutic agents [227,230].

6.3 Strengths and limitations

Even though this is one of the largest clinical cohorts reported in T-DLBCL, the number of patients in this study is still relatively low, which should be taken into account when interpreting the results of this study. Limitations of the Nordic collaborative study also include the retrospective analysis of the data and the potential patient selection bias caused by this approach. Due to the retrospective nature of the study, the patient material and their treatments are heterogeneous. The findings on the benefit of different treatment modalities need to be interpreted with caution since the relapse rate might be underestimated among the most fragile patients not necessarily actively diagnosed with a potential lymphoma relapse. Additionally, patients with the most comorbidities were most likely not treated with the most intense treatment modalities, possibly further impairing the results on the efficacy of the less toxic treatment approaches. As the overall incidence of CNS relapses was low in our patient material, evaluation of the possible effect of different treatment modalities on the risk of CNS relapse is very challenging.

The advantages of the biological studies presented in this thesis include the considerably high number of T-DLBCL patients with primary diagnostic tumor tissue available for the analysis compared to the previously published biological data on T-DLBCL. In addition, the mIHC technology used in this study provided us the opportunity of characterizing distinct immune cell subtypes, their immunophenotypes, and the localization of immune checkpoint and exhaustion markers more specifically than traditional IHC. The restrictions include the lack of functional analysis of different immune cell phenotypes and their interactions in the TME as well as limitations caused by the rareness of this malignancy. The number of patients treated with current standard of care including immunochemotherapy is inevitably low in the biological studies of this thesis, not necessarily enabling us to detect all significant associations of distinct immune cell subtypes and their immunophenotypes with clinical factors and patient outcome. Additionally, our data provides information on the TME of T-DLBCL at the time of diagnosis but leaves out the knowledge on possible changes in the contribution and role of the immune cells during lymphoma treatments.

6.4 Future perspectives

With the limitations related to the rareness of T-DLBCL, the possibility of evaluating different treatment modalities in a randomized phase III trial are limited, and the currently recognized standard of care seems justified based on several retrospective analyses and few phase II trials. The results from our Nordic collaborative study support the current treatment policy and highlight the importance of IV CNS-targeted chemotherapy. Future perspectives concerning clinical studies should, however, evaluate possible less toxic treatment modalities for the elderly more fragile patients not eligible for the current standard of care with an intense chemotherapy approach.

The findings of our biological studies give significant novel information on the genetic landscape as well as tumor-infiltrating immune cell subtypes, their immunophenotypes, distribution, and correlation to known clinical and biological risk factors and survival in T-DLBCL. Within the last years, the therapeutic landscape of both solid tumors and lymphomas has dramatically changed with the introduction of several new targeted therapies, including checkpoint inhibitors targeting the PD-1–PD-L1 pathway [472,503]. Based on preliminary results, the PD-1 Ab nivolumab seems promising also in T-DLBCL, and further studies are ongoing [476]. Abs targeting inhibitory receptors TIM-3 and LAG-3 have also been studied, and the findings suggest that the function of these receptors is reversible [251,339,348,496,504]. Based on data provided by our studies, future clinical trials should include patients with T-DLBCL in studies evaluating the effectiveness of these Abs. Furthermore, as mAbs are commonly much more well tolerated than chemotherapy, this might be one solution for providing effective personalized medicine treatment approaches also for elderly, more fragile patients in the future.

Future perspectives on biological studies should focus on further characterizing the functional role of distinct immune cell subtypes in the TME of T-DLBCL. Studying cell-to-cell interactions between T_{reg}s and other host immune cells such as TAMs and Th1 cells would be one interesting approach. The impact of treatments, especially immune checkpoint inhibitors, on the contribution and role of immune cells in the TME would bring important new information, and would require repetitive tumor biopsies also after and in between the treatments. Interactions between tumor cells and the host immune cells might also be different in T-DLBCL compared to other, primary DLBCLs presenting at sites that are not immune-privileged. As PCNSL is known to share several biological features with T-DLBCL and as the CNS relapse rate of T-DLBCL is known to be significantly high, another interesting aspect in the future would be to study the genetic landscape and the possible interactions between tumor cells and different host immune cells in tissue samples of T-DLBCL patients' CNS relapses and relapses outside of the CNS and the contralateral testis. Comparing the results with findings in PCNSL could perhaps further widen our understanding of these rare and unique lymphoma subtypes presenting at immune-privileged sites.

7 Summary

T-DLBCL is a rare and aggressive lymphoid malignancy with dismal prognosis and a high recurrence rate. Due to the lack of prospective randomized clinical trials the evidence on different treatment modalities has been frail. The role of the TME, more specifically TAMs, TILs, and T-cell exhaustion, has been uncharacterized. The aim of this study was to evaluate the treatment results of T-DLBCL patients and to characterize the role and prognostic value of the TME in T-DLBCL.

1. The results of this thesis support the use of the currently recognized standard of care with anthracycline-based immunochemotherapy combined with IV CNS-targeted chemotherapy and prophylactic treatment of the contralateral testis as the first line treatment of T-DLBCL patients.
2. T-cell inflamed TME associates with favorable survival in T-DLBCL.
3. The content of different TIL immunophenotypes varies between T-DLBCL patients. Higher content of TILs expressing PD-1 associates with longer survival, while subpopulations of T_{reg}s predict worse outcome of patients with T-DLBCL.
4. High content of PD-1$^+$ TILs correlates with the proportions of PD-L1$^+$ TAMs and translates into longer survival of patients with T-DLBCL.

Future studies are needed to further characterize the functional roles of distinct tumor-infiltrating immune cells in T-DLBCL, and to evaluate novel and targeted therapeutic options also for elderly, more fragile patients with T-DLBCL.

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